THE

BIOCHEMICAL JOURNAL

CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, MANAGER

LONDON
FETTER LANE, E.C. 4

EDINBURGH
100 PRINCES STREET

LONDON: H. K. LEWIS AND CO., LTD., 136 Gower Street, W.C. 1
LONDON: WILLIAM WESLEY AND SON, 28 Essex Street, Strand, W.C. 2
CHICAGO: THE UNIVERSITY OF CHICAGO PRESS
BOMBAY, CALCUTTA, MADRAS: MACMILLAN AND CO., LTD.
TORONTO: J. M. DENT AND SONS, LTD.
TOKYO: THE MARUZEN-KABUSHIKI-KAISHA

All rights reserved

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

EDITORIAL COMMITTEE

Dr E. F. ARMSTRONG
Prof. V. H. BLACKMAN
Prof. A. J. BROWN
Mr J. A. GARDNER
Prof. F. G. HOPKINS
Prof. F. KEEBLE
Prof. B. MOORE
Prof. W. RAMSDEN
Dr E. J. RUSSELL

VOLUME XI 1917

CAMBRIDGE
AT THE UNIVERSITY PRESS
1917

Vol. XI, No. 1 May 1917

JUN 21 1917
UNIV. OF MICH. LIBRARY

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

EDITORIAL COMMITTEE

DR E. F. ARMSTRONG
PROF. V. H. BLACKMAN
PROF. A. J. BROWN
MR J. A. GARDNER
PROF. F. G. HOPKINS
PROF. F. KEEBLE
PROF. B. MOORE
PROF. W. RAMSDEN
DR E. J. RUSSELL

CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, Manager
LONDON: Fetter Lane, E.C. 4
EDINBURGH: 100, Princes Street

also

H. K. LEWIS & Co., Ltd., 136, Gower Street, London, W.C. 1
WILLIAM WESLEY & SON, 28, Essex Street, Strand, London, W.C. 2
CHICAGO: The University of Chicago Press
BOMBAY, CALCUTTA AND MADRAS: Macmillan and Co., Ltd.
TORONTO: J. M. Dent & Sons, Ltd.
TOKYO: The Maruzen-Kabushiki-Kaisha

1917

Price Seven Shillings net.

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

The annual subscription is 25*s*., which includes a copy of the Journal, and becomes due on January 1st. Further information may be obtained on application to the Hon. Sec., Dr R. H. Plimmer, University College, London, W.C. 1, to whom subscriptions should be sent.

Papers for publication should be sent to Prof. W. M. Bayliss, F.R.S., University College, W.C. 1, or to Prof. A. Harden, F.R.S., Lister Institute, Chelsea Gardens, S.W. 1. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued quarterly and the date at which each paper is received by the editors is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£1. 1*s*. net per volume (post free) payable in advance) should be addressed to Mr C. F. Clay, Cambridge University Press, Fetter Lane, London, E.C. 4.

Volumes VII–IX (1913–1915). In four parts, paper covers, 21*s*. net per volume; bound in Buckram, 25*s*. net per volume. Vol. X (1916), containing Index to Volumes I–X, in five parts, paper covers, 24*s*. 6*d*. net; bound in Buckram, 30*s*. net. Quotations for Vols. I–VI will be sent on application.

The Cambridge University Press has appointed the University of Chicago Press agents for the sale of the *Biochemical Journal* in the United States of America and has authorised them to fix the following subscription price. $5.25 net.

Notice to Contributors.

1. Contributors receive 50 copies of their papers free and can purchase additional copies if notice be given when their proofs are returned.

2. Papers forwarded to the Editors for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

3. All communications intended for publication must be type-written.

4. **References.** References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann (1912, 1), *Zeitsch. Biol.* 59, 17. In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus [Ackermann, 1912, 1]. If reference is made to several papers published by one author in a single year, these should be numbered in sequence and the number quoted along with the year both in the text and in the collected references.

5. Chemical formulae should be written, as far as possible, in a single horizontal line.

6. Illustrations and curves accompanying the papers must be carefully drawn, about twice the size of the finished block, on smooth white Bristol boards in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Further information can be obtained from the Editors.

CONTENTS

No. 1 (MAY)

PAGE

I. Occurrence of Raffinose in the Seed of the Jute Plant (*Corchorus capsularis*). By H. E. ANNETT 1

II. Accessory Factors for Plant Growth. By O. ROSENHEIM. (With Plate I) 7

III. The Preparation of Desoxycholic Acid. By W. MAIR . . 11

IV. Adsorptive Stratification in Gels, II. By S. C. BRADFORD . 14

V. The Reaction of Sera as a Factor in the Successful Concentration of Antitoxic Sera by the Methods at present in use. By A. HOMER. (With Two figures) 21

VI. Further Contributions to the Technique of Preparing Membranes for Dialysis. By W. BROWN 40

VII. The Synthesis of Racemic *Pr*-2-Methyltryptophan. By G. BARGER and A. J. EWINS 58

VIII. The Conditions of Activation of Washed Zymin and the Specific Function of Certain Cations in Alcoholic Fermentation. By A. HARDEN 64

IX. On a New Type of Chemical Change produced by Bacteria. The Conversion of Histidine into Urocanic Acid by Bacteria of the Coli-Typhosus Group. By H. RAISTRICK 71

No. 2 (AUGUST)

X. On the Oxidation of Amino-acids and of related substances with chloramine-T. By H. D. DAKIN 79

XI. A rapid process for the estimation of the Higher Fatty Acids and Soaps in Faeces. By J. S. SHARPE 96

XII. The distribution of Nitrogen in Beer. By J. S. SHARPE . 101

XIII. The Viscosity of Solutions. By S. ARRHENIUS 112

XIV. The estimation of Succinic Acid. By E. C. GREY . . . 134

XV. The distribution of the Fatty Acids in the Milk Fat of the Cow and Sheep. By C. CROWTHER and A. HYND 139

XVI. The Oxidation of the Alkali Butyrates by Hydrogen Peroxide with the Production of Succinic Acid. By E. CAHEN and W. H. HURTLEY 164

XVII. The alleged Antineuritic Properties of α-Hydroxypyridine and Adenine. By A. HARDEN and S. S. ZILVA 172

XVIII. Colour Changes produced by two groups of Bacteria upon Caseinogen and certain Amino-acids. By E. C. V. CORNISH and R. S. WILLIAMS 180

329860

Nos. 3 & 4 (December)

PAGE

XIX. L'Acide lactique est-il un produit intermediaire de la Fermentation Alcoolique? Par Alexandre Lebedev 189

XX. Contributions to the Biochemistry of Pathogenic Anaerobes. II. The acid production of *Bacillus Welchii* (*B. perfringens*) and *Bacillus sporogenes* (Metchnikoff). By C. G. L. Wolf and S. V. Telfer. (With Two figures) 197

XXI. Contributions to the Biochemistry of Pathogenic Anaerobes. III. The effect of Acids on the Growth of *Bacillus Welchii* (*B. perfringens*) and *Bacillus sporogenes* (Metchnikoff). By C. G. L. Wolf and J. E. G. Harris. (With Eight figures) 213

XXII. The Nitrogenous Extractives of Tumours. By J. C. Drummond 246

XXIII. A Study of the Water-Soluble Accessory Growth promoting Substance in Yeast. I. By J. C. Drummond. (With Fifteen charts) . 255

XXIV. The Reduction of Phenylethylamine. By A. Weinhagen 273

XXV. Further Observations on the Influence of Phenol and of Cresylic Acid on the Concentration of Antitoxic Sera by the Banzhaf (1913) Process. By A. Homer 277

XXVI. A Note on the use of Indicators for the Colorimetric Determination of the Hydrogen Ion Concentration of Sera. By A. Homer . 283

XXVII. On the Influence of the Heat Denaturation of Pseudoglobulin and Albumin on the Nature of the Proteins appearing in Concentrated Antitoxic Sera. By A. Homer. (With Two figures) . . 292

XXVIII. The Formation of Creatine. Effects on the Excretion of Creatine in the Bird produced by Paraformaldehyde and Hexamethylene-Tetramine given separately and combined with Arginine Carbonate and other Substances. By W. H. Thompson. (With an Addendum by E. A. Werner) 307

XXIX. The Preparation of Plant Nucleic Acids. By G. Clarke and S. B. Schryver 319

XXX. A Comparative Study of Tumour and Normal Tissue Growth. By J. C. Drummond. (With Thirty-one charts) 325

I. OCCURRENCE OF RAFFINOSE IN THE SEED OF THE JUTE PLANT (*CORCHORUS CAPSULARIS*).

By HAROLD EDWARD ANNETT.

From the Agricultural Research Laboratory, Dacca, Bengal.

(*Received December 29th, 1916.*)

Raffinose has been shown to occur in beet molasses, cotton seed, barley grains, wheat sprouts and eucalyptus manna. It is now shown to be present in jute seeds.

Preparation. In the course of an examination of jute seed for a glucoside Mr R. S. Finlow, Fibre Expert to the Government of Bengal, obtained a substance, which had a sweet taste, and handed the material to me for further investigation. The jute seed had been finely ground and then exhausted with ether and with petrol. The residue was next exhausted with alcohol. From this alcoholic extract the substance under investigation was obtained as a copious white precipitate on the addition of ether.

The impure substance was dissolved in hot 80 % alcohol. On filtering and cooling, the solution deposited after several days rosettes of white needles. The crystals were collected on a filter and recrystallised from 80 % alcohol. About 3 g. of the pure crystals were finally obtained.

General Physical and Chemical Properties.

The substance had an extremely sweet taste, was very soluble in water, and crystallised from alcohol in characteristic rosettes of white needles. It gave no osazone and did not reduce Fehling's solution. 10 cc. of a 1 % solution gave only 2·5 milligrams of CuO under Brown and Morris's conditions. On hydrolysis with acid or treatment with emulsin or invertase, reducing sugars were formed and then an osazone was readily obtained. It gave Selivanoff's reaction and on boiling with concentrated hydrochloric acid gave a red colour indicating the presence of fructose.

Water of crystallisation. On heating a small portion of the crystals in the steam oven they melted indicating the presence of much water of crystallisation. Some freshly prepared crystals were well washed with absolute alcohol on a small Hirsch funnel under suction and then dried thoroughly between filter paper. About 0·5 g. was then dried *in vacuo* in a toluene bath in presence of phosphorus pentoxide. For the first few hours the temperature was kept below 60° C. but it was finally increased to 105° C. and maintained at that temperature until a constant weight was obtained. This took about 24 hours.

0·5420 g. substance gave water of crystallisation = 15·09 %. For pure raffinose the theory is 15·15 %. A sample of recrystallised raffinose gave 15·54 %.

Specific rotatory power. This had to be determined in a Schmidt-Haensch saccharimeter using ordinary white light. The substance showed no mutarotation.

0·4602 g. of anhydrous sugar was dissolved in water and made up to 50 cc. This gave a reading on the sugar scale of + 6·64. Raffinose should have given + 6·51. The agreement is within the error due to the unfavourable conditions under which the rotation had to be determined.

Action of enzymes. These tests were carried out in conjunction with parallel tests on pure raffinose.

(*a*) *Emulsin.* When emulsin acts on raffinose, cane sugar and galactose are produced according to the equations:

$$C_{18}H_{32}O_{16} \cdot 5H_2O \rightarrow C_6H_{12}O_6 + C_{12}H_{22}O_{11}.$$

Whence 100 parts of raffinose give 30·3 parts of galactose and 57·57 parts of saccharose.

Since $[\alpha]_D^{20}$ for raffinose = + 104°, for galactose = + 81°, and for sucrose = + 66·5°.

30·3 % galactose will give a rotation of 0·303 × 81 = + 24·54°,

57·57 % sucrose will give a rotation of 0·5757 × 66·5 = + 38·28°,

$\therefore$ $[\alpha]_D^{20}$ of the mixture will give a rotation of = + 62·82°,

the $[\alpha]_D^{20}$ falling from + 104° to + 62·82°.

Exp. 1. 1·0103 g. of pure raffinose were dissolved in water and the volume made up to 100 cc. This gave a reading of + 5·94.

To 50 cc. of this solution 0·1 g. emulsin + 0·5 cc. toluene were added. The reading became + 3·50 (corrected for emulsin).

Exp. 2. 1·2280 g. of the jute sugar in the anhydrous condition were dissolved in water and the volume made up to 100 cc. This gave a reading

in the saccharimeter of + 8·51. To 50 cc. 0·2 g. emulsin and 0·5 cc. toluene were added. The reading became + 5·32 (corrected for emulsin).

The reading of the pure raffinose solution after emulsin action should have been 62·8/104 × 5·94 = + 3·59, and that of the jute sugar 62·8/104 × 8·51 = + 5·14.

It must be noted here that with the emulsin used some 10 days were required before a constant saccharimeter reading was obtained.

(*b*) *Invertase + Melibiase.* When invertase acts on raffinose, d-fructose and melibiose are produced.

It can readily be shown that the $[\alpha]_D^{20}$ of raffinose + 104° falls to + 54·4° as a result of the action of invertase. The action of the melibiase in addition to the invertase, produces from one molecule of raffinose, one molecule each of d-glucose, d-galactose and d-fructose. Hence it follows that the $[\alpha]_D^{20}$ of raffinose should fall after the action of invertase + melibiase from + 104° to + 12·5°.

The invertase used in these tests was prepared according to Hudson's method [1914] which gives a highly pure and active product which even in this climate has retained its activity for more than a year. It was made from bottom yeast and contained melibiase.

Exp. 3. To 30 cc. of the pure raffinose solution which had been treated with emulsin (Exp. 1), 0·25 cc. of invertase + melibiase solution was added together with a few drops of toluene. A further fall in rotation was observed and no further fall took place after 24 hours. The original reading of the raffinose solution was + 5·94, the action of emulsin reduced the reading to + 3·50, and the action of invertase further reduced the reading to + 0·87 (corrected for invertase control).

Theoretically the final reading should have been 12·5/104 × 5·94 = + 0·71.

Since the readings were made at about 30°, it is to be expected that the actual reading would be too high owing to the large increase in $[\alpha]_D^{20}$ of fructose caused by rise of temperature.

Exp. 4. 0·9320 g. of roughly anhydrous jute sugar was dissolved and made up to 100 cc. The solution gave a reading in the saccharimeter (200 mm. tube) of + 6·80. To 60 cc. of this solution, 1 cc. of invertase + melibiase solution and 0·5 cc. of toluene were added and the mixture incubated at 37°. The rotation became constant at + 0·94 (corrected for invertase) after 48 hours.

Assuming the sugar to be raffinose this final reading should have been 12·5/104 × 6·80 = + 0·85.

Osazone formation. Exp. 5. 20 cc. of a 1 % solution of the jute sugar were mixed with 0·4 g. of phenylhydrazine hydrochloride and 0·6 g. of sodium acetate and heated in the water bath. No osazone was obtained after 1 hour even on cooling. 0·411 g. of partly dehydrated jute sugar was dissolved in about 4 cc. of water, 0·5 cc. invertase and a few drops of toluene were added, and the mixture incubated at 30° for two days. 5 cc. of a solution containing 1 g. phenylhydrazine hydrochloride and 1·5 g. sodium acetate were then added.

The mixture was heated in a water bath. Under these conditions fructose should form an osazone in 3 minutes, glucose in 4—5 minutes and galactose in 15—19 minutes [1912].

An osazone formed in 3 minutes. After 15 minutes the liquid was filtered and the osazone recrystallised twice from alcohol (Crop 1).

The filtrate was again heated in the water bath for 1 hour when a second crop of crystals was filtered off. These were recrystallised twice from alcohol to form Crop 2. The filtrate was again heated for $\frac{3}{4}$ hour and a third crop of crystals was obtained and recrystallised twice from alcohol (Crop 3).

As a control 0·15 g. each of galactose, glucose and fructose were dissolved in about 4—5 cc. of water and 5 cc. of the phenylhydrazine hydrochloride and sodium acetate mixture added. Three crops of crystals were obtained exactly as with the jute sugar.

Crop 3 in each case was very small in amount.

The melting points of these crops of crystals were taken. Duplicate determinations being done in each case.

They were found to be:

Crop 1. M.P. (*a*) 203, (*b*) 202°.
Crop 2. M.P. (*a*) 202·5, (*b*) 203°·5.
Crop 3. M.P. (*a*) 196·5, (*b*) 195°·5.

The melting points of the crystals from the mixture of the sugars were (corrected):

Crop 1. M.P. (*a*) 204, (*b*) 203°.
Crop 2. M.P. (*a*) 201·5, (*b*) 203°·5.
Crop 3. M.P. (*a*) 193·5, (*b*) 194°·5.

Pure glucosazone under the same conditions had a M.P. (corrected) of 205 to 206°. The M.P. of galactose d-phenylosazone is given as 194—196° [1912].

The crystals were also examined microscopically, after mounting in xylene balsam.

The crystals of Crop 1 from the jute sugar were all of one kind collected

into the characteristic bundles of needles generally seen with phenylglucosazone.

Those of Crop 2 resembled Crop 1, but showed a few square ended yellow leaflets.

Those of Crop 3 consisted mostly of square ended bright yellow leaflets.

The microscopic appearance of the crops of crystals from the artificial mixture of sugars agreed in appearance with that of Crops 1, 2, 3 obtained from jute sugar.

The above experiments prove that the sugar obtained from jute seed is raffinose. By way of confirmation a 1 % solution of the sugar was treated with emulsin in presence of toluene. Emulsin hydrolyses raffinose to galactose and cane sugar. After 10 days the liquid was heated with phenylhydrazine hydrochloride and sodium acetate. An osazone was produced which contained many yellow square ended platelets characteristic of galactose phenylosazone.

The amount of raffinose in jute seed. 200 g. of finely ground seed were well shaken with 500 cc. distilled water, containing 10 cc. toluene, for one hour and filtered.

To 50 cc. of the filtrate 1 cc. of basic lead acetate was added and the liquid filtered. A bright solution was obtained from which lead was removed by means of solid sodium phosphate. This gave a reading in the 100 mm. tube of the saccharimeter of + 8·1.

25 cc. of this solution were slightly acidified by the addition of four drops of acetic acid and then 0·5 cc. invertase + melibiase and 0·5 cc. toluene were added and the mixture incubated at 37° for two days. Four drops concentrated NH_4OH were then added and the liquid filtered. A reading of + 5·2 was obtained in the 100 mm. tube of the saccharimeter.

To 25 cc. of the original water extract (which was faintly acid) 0·5 cc. invertase + melibiase and 0·5 cc. toluene were added and it was then incubated as above. After two days 0·5 cc. of basic lead acetate was added, the liquid being filtered after shaking. Lead was separated from the filtrate with sodium phosphate and the filtrate gave a reading in the 100 mm. tube of + 5·2.

The invertase had thus caused a fall in rotation, measured in the 100 mm. tube of + 8·1 to 5·2 or 2·9 degrees on the sugar scale. For the 200 mm. tube this equals 5·8.

The invertase and melibiase solution will cause a fall in rotation for raffinose of 91·5° from + 104° to + 12·5°.

Assuming that raffinose is the only sugar present in the jute seed capable

of being acted on by invertase, the original rotation due to raffinose for the 200 mm. tube would have been $5{\cdot}8 \times 104/91{\cdot}5 = 6{\cdot}6$; this corresponds to 2·29 angular degrees and gives a content of about 1·1 g. raffinose per 100 cc. of the original water extract from 200 g. jute seed. Since there were 500 cc. of water added; the jute seed contains about 2·25 % raffinose.

Conclusions.

Raffinose has been shown to occur in the seed of the jute plant *Corchorus capsularis*. In the particular seed sample examined, the sugar occurs to the extent of about 2·25 %.

REFERENCES.

Browne, C. A. (1912), *A Handbook of Sugar Analysis*, London, 1912.
Hudson, C. S. (1914), *J. Amer. Chem. Soc.* **36**, 1566.

II. ACCESSORY FACTORS FOR PLANT GROWTH.

By OTTO ROSENHEIM.

From the Physiological Laboratory, King's College, London.

(*Received January 4th, 1917.*)

In his endeavours to find a suitable medium for the growth of the nitrogen-fixing bacteria of the "Azotobacter" group, Prof. Bottomley observed that although these organisms failed to grow in natural peat, they flourished when grown in sphagnum peat which had previously been "rotted" by the agency of ordinary aërobic soil organisms. Sterilised rotted peat, inoculated with a mixed culture of nitrogen-fixing bacteria was subsequently found to produce striking results in the development of pot grown plants, results which could not be ascribed to the manurial contents of the treated peat nor to the beneficial effect of the development of the nitrogen-fixing bacteria. It was found later, indeed, that the presence of these bacteria was not an essential factor. Prof. Bottomley was at a loss to explain these results, which had been confirmed by impartial experienced horticulturists, and in discussion with me suggested an examination of the chemical composition of the treated peat.

Before entering into this necessarily lengthy investigation I desired to convince myself of the activity of the material. In order to eliminate a possible beneficial effect on the soil porosity, etc., due to the increased water-retaining power of the peat-soil mixture, I decided to use an aqueous extract of the material. Although the actual manurial properties of the treated peat are small and its contents of nitrogen, phosphorus and potassium are low as compared with any ordinary fertiliser, I purposely chose an amount of the extract which should exclude its possible manurial effect.

As it was hardly to be expected that the small quantity used in the first experiment could produce any effect, it was my original intention gradually to raise the amount until the minimal effective dose was reached. The surprising result of the experiment to be described, however, made this unnecessary

and led me to the assumption that a substance is present in the treated peat which plays a rôle in plant growth similar to that played by the now generally accepted food accessories in animal growth. Owing to various causes I had no opportunity of continuing this work, and as my experiments, which formed the starting point of Prof. Bottomley's subsequent researches, were necessarily only briefly alluded to by him, a somewhat fuller account seems desirable at this stage.

My experiments were begun in the late autumn of 1913, a season unfavourable for plant growth under ordinary conditions and for that reason more likely to show the effect of any stimulating agent. The only plants I had available at that time for experiments were a number of seedlings of *Primula malacoides*. It was recognised that this primula is by no means an ideal plant for this work, as it is well known to practical gardeners that a considerable variation in its character and rate of growth may occur. An experimental error due to this cause was guarded against by selecting from a large batch of seedlings a considerable number of plants of apparently equal development and by beginning the treatment only at such a relatively advanced stage of their growth that any inequality in their character would have been noticeable. The seedlings were raised during the summer, 1913, and a large number of them pricked out into boxes. Some of the plants were potted up on Oct. 5th in ordinary potting compost (loam, leaf-mould and sand). Shallow porous pots, so-called dwarf alpine pots, of uniform size (12·5 cm. diam. and 500 cc. capacity) were used. A layer of 1·5 cm. washed granite chips served as drainage. On Oct. 24th a series of twelve plants were selected which were as far as possible in a uniform stage of development. Six of these were used as controls, whilst the other six were watered with 60 cc. each of the diluted peat extract (see below). The plants were kept under the ordinary conditions in an unheated greenhouse. During the course of the experiment the outside temperature fell only on a few occasions below freezing point, without affecting the development of the plants.

No difference could be detected between the treated and untreated plants during the first three weeks, and therefore on Nov. 15th three of the treated plants received another dose of 60 cc. of the extract[1]. During the fifth week, however, the difference became very marked indeed. The treated plants not only grew considerably taller, but showed a remarkable luxuriance

[1] It may be mentioned that this second treatment did not produce any additional beneficial effect, an observation which speaks in favour of the view of "accessories."

of growth. They were of a much deeper green and had larger leaves. They produced flower stems in greater abundance and also the size of the individual flowers and the number of whorls was increased. One each of the plants of each series was photographed on Dec. 7th, Pl. 1, Fig. 1, 14th, Fig. 2, and 26th, Fig. 3, and the last photograph shows clearly the increased development of the root system which corresponds to the increase in the foliage. The effect was uniform on all the plants of the series and the control plants also were very uniform in their behaviour.

For the preparation of the extract 15 g. of treated peat, which was kindly put at my disposal by Prof. Bottomley, was left standing for 18 hours with 2 litres of tapwater. 500 cc. of the brown clear solution were decanted and diluted to 1250 cc. The extract contained:

0·045 % Total Solids, 0·011 % Inorganic Substances,
0·034 % Organic Substances, 0·003(2) % Nitrogen.

The plants received therefore in one application the extract of 0·18 g. of treated peat containing 20 mg. of organic matter and 1·9 mg. of nitrogen, i.e. roughly 4 per million nitrogen to soil.

The effect produced on plant growth by this small quantity of soluble substances suggested to me an analogy to that due to "vitamines" or accessory food substances in animal growth and led me to investigate the behaviour of the peat towards reagents which are known to dissolve or precipitate "vitamines." Some of these substances are soluble in alcohol and are precipitated, or carried down in an adsorbed condition, by phosphotungstic acid.

Extracts were made for comparison from garden soil, natural peat and treated peat by allowing 15 g. of each of the materials to stand for one hour with 100 cc. of water. To 20 cc. of each filtrate 2·5 cc. conc. H_2SO_4 in 10 cc. water was added and the volume made up to 50 cc. A brown floccular precipitate of humic acid formed in the treated peat extract and was filtered off. Equal volumes of a 30 % phosphotungstic acid solution were added to each filtrate. The extract from treated peat gave an immediate copious precipitate, whilst that of natural peat only gave a faint opalescence and the extract of garden soil remained clear.

Extracts were made from the materials in a similar way with boiling alcohol or acetone. The solvent was evaporated and the residue taken up with water. Again a heavy precipitate formed on adding phosphotungstic acid to the extract of the treated peat. It was further noted that a considerable part of the phosphotungstic acid precipitate was insoluble in acetone,

a fact which may be of use for the isolation of the active substance [see Funk, 1916].

It had been suggested by Funk and Macallum [1913] that the blue colour reaction which is given by Folin and Macallum's uric acid and phenol reagents, may serve as an index for the presence of "vitamines" [see also Williams and Seidell, 1916]. I was unable to test the original extract used for these experiments in this direction. A fresh supply of treated peat, whose activity on plants had not, however, been tested and was considered doubtful, did not give the uric acid reaction, but developed a blue colour with the phenol reagent. Prof. Bottomley has since stated that active preparations show the uric acid reaction distinctly.

The chemical examination of the treated peat extract lends therefore strong support to my view which was arrived at from its effect on plant growth and demonstrated the presence of substances similar to the "vitamines" in their general behaviour. At my suggestion the action on plant growth of alcoholic extracts and the fractions obtained from them by phosphotungstic acid were tested in Prof. Bottomley's laboratory. It was found that the active substances were contained in the alcoholic extract and its sub-fractions, and that in minute quantities they exerted a remarkable stimulating effect on plant growth (Bottomley, 1915, 1, 2).

As far as the activity of the treated peat is concerned these observations therefore supply a working hypothesis which is being further tested experimentally. They suggest besides many interesting problems regarding plant nutrition in general, and I am at present engaged in some experiments on the effect on plant growth of the substances contained in yeast and milk, which are known to possess growth promoting properties in animals.

REFERENCES.

Bottomley, M. A. (1915, 1), *Proc. Roy. Soc. B.* **88**, 237.
—— (1915, 2), *Proc. Roy. Soc. B.* **89**, 102.
Funk, C. (1916), *Biochem. Bull.* **5**, 1.
—— and Macallum, A. B. (1913), *Biochem. J.* **7**, 356.
Williams, R. R. and Seidell, A. (1916), *J. Biol. Chem.* **26**, 431.

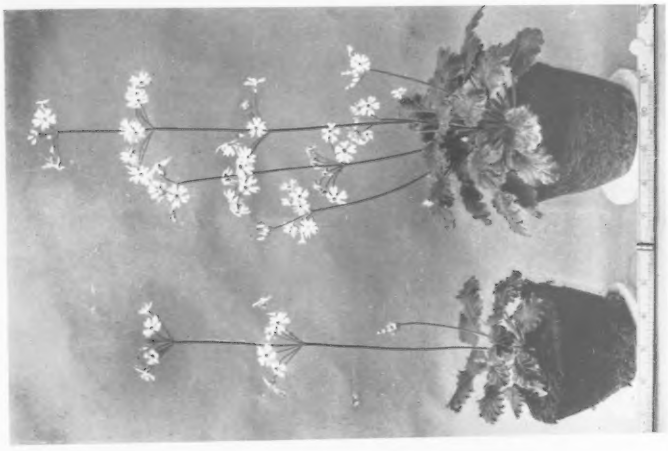


Fig. 3

Fig. 2

Fig. 1

III. THE PREPARATION OF DESOXYCHOLIC ACID.

By WILLIAM MAIR.

From the Research Laboratory of the Metropolitan Asylums Board, London.

(*Received January 22nd, 1917.*)

In investigating the solvent action of bile salts on "pneumococci" it was found that sodium desoxycholate is ten times as active, in this respect, as sodium cholate, or commercial sodium taurocholate. The remarkable activity of the desoxycholate in this connection makes its introduction into bacteriological technique desirable, and, as it is possible that the salt may also prove to be of value in the prevention and treatment of local pneumococcal infections, it appears to be worth while to place on record a method of preparation of desoxycholic acid which seems to offer certain advantages over those at present in use. The method is in part suggested by the recent work of Wieland and Sorge [1916] who have shown conclusively that the so-called "choleic" acid is a peculiar combination of desoxycholic with stearic (or other fatty) acid, the proportion of the latter being so small that the difference in composition is not appreciable in an elementary analysis.

It is well known that desoxycholic acid can be purified by recrystallisation from glacial acetic acid, and the chief feature of the present method is that this solvent is applied directly to the crude mixture of bile acids.

In detail the method is as follows:—To four litres of ox bile 240 g. of caustic soda are added and dissolved by stirring and heating. The alkaline bile is then boiled gently in an iron digester for about 20 hours. A certain amount of evaporation takes place and the volume should now be between 2 and 3 litres. While still hot it is made neutral to phenolphthalein by adding gradually about 350 cc. strong hydrochloric acid. A flocculent precipitate of silica forms but none of the bile acid is thrown down, provided the solution is sufficiently warm. The amount of silica in ox bile is quite appreciable, amounting to one or two grams per litre. After cooling the bile is filtered. The filtrate is rendered acid to litmus by adding about 50 cc. glacial acetic

acid. The bile acids separate out in a fluid crystalline mass which is at first white but rapidly absorbs pigment. By gentle rotation of the flask the bile acids can be made to adhere in a single mass and can easily be separated by decantation of the mother liquor. The fluid crystalline mass consists of the mixed bile acids holding acetic acid in combination. It is squeezed to get rid of excess of water, and it can then be handled like a mass of putty and so transferred to a flask of about one litre capacity. Rather more than an equal volume of glacial acetic acid (about 600 cc.) is added, and the flask is warmed on the water bath until the whole is dissolved. The solution is poured into a beaker and allowed to stand until crystallisation is complete, usually two or three days. By this procedure a crystallisation of the bile acids from acetic acid of about 60 % strength is obtained. The mixed crystals contain practically all the desoxycholic acid; they also contain an almost equal amount of cholic acid and some fatty acid. The crystals are filtered off, washed free from pigment with 60 % acetic acid, sucked as dry as possible on the filter, and re-dissolved by heating with 300 cc. glacial acetic acid. The solution is now only slightly pigmented, but contains traces of insoluble matter, probably silica. The hot solution is therefore filtered and allowed to stand. The crystals which form now consist chiefly of desoxycholic acid with some admixture of cholic acid. They are separated and pressed dry between porous tiles. They are then dissolved by boiling in about 750 cc. 60 % acetic acid and the hot solution is filtered into a beaker; sometimes a little oily substance remains undissolved and floats on the surface (fatty acid), this can easily be removed after it solidifies on cooling. Next day the crystals are filtered off, washed first with 60 % acetic acid and then thoroughly with water, sucked dry, and dried completely in the hot air oven at 100°. A perfectly white crystalline substance with M.P. between 170° and 175° is thus obtained. It is practically pure desoxycholic acid (M.P. 172°—173°). The yield from four litres of bile was 32 g. with M.P. 172°—173°; from another four litres of bile the yield was 42 g., M.P. 170°.

To prepare the acid from commercial taurocholate, 500 g. of the latter are dissolved in 3 litres of water with 200 g. of caustic soda and the further procedure is as above. The yield from 1000 g. of the taurocholate was 50 g. with M.P. 173°—174°.

As a general procedure for the isolation of the bile acids the method is only recommended where a maximum yield of desoxycholic acid is desired and where it is not necessary to recover a large amount of cholic acid. The cholic acid can, however, be recovered from the purer mother liquors. The

acetic acid is distilled off as completely as possible, finishing the distillation *in vacuo* on the water bath. The residue is treated with a little hot alcohol, and aqueous caustic soda is added till the solution is just alkaline to phenophthalein. The alcohol is boiled off and more water is added. After cooling the cholic acid is precipitated by adding dilute hydrochloric acid. It separates in a fluid crystalline mass containing acetic acid to which it obstinately clings. After pressing out the mother liquor the cholic acid is subjected to prolonged digestion with hot water to remove the acetic acid. On cooling it forms a hard cake which still smells of acetic acid. This is broken up and powdered in a mortar and then dried, first in an exhausted desiccator over caustic soda, and then in the hot air oven at 100°. It is then dissolved in rather more than twice its volume of absolute alcohol by boiling, and is purified by recrystallisation from alcohol.

From the acetic acid mother liquors (second and third recrystallisations) corresponding to 75 g. of desoxycholic acid there was obtained 50 g. of cholic acid and 7 g. of fatty acid.

These figures therefore give the approximate composition of the crystals obtained from the first acetic acid crystallisation from the crude bile acids.

Where a maximum yield of cholic acid is desired it is better to follow Schryver's [1912] procedure as far as the precipitation with magnesium chloride. The insoluble magnesium desoxycholate is then filtered off from the hot solution, sucked dry on the filter and dissolved in hot glacial acetic acid. On cooling and standing desoxycholic acid crystallises out, and the further procedure is as already described.

I wish here to express my indebtedness to Dr Schryver for his kindness in supplying me with samples of the various bile acids prepared by his method.

REFERENCES.

Schryver (1912), *J. Physiol.* **44**, 265.
Wieland und Sorge (1916), *Zeitsch. physiol. Chem.* **97**, 1.

IV. ADSORPTIVE STRATIFICATION IN GELS, II.

By SAMUEL CLEMENT BRADFORD.

(*From the Science Museum, S. Kensington, S.W.*)

(*Received February 1st, 1917.*)

It has been postulated [Bradford, 1916] that the formation of stratified deposits in gels may be explained by the action of the finely divided particles of the precipitate in adsorbing the solute from the gel in their immediate neighbourhood. The accompanying phenomena are, however, so numerous and of so varied a nature that it is necessary to examine them more in detail and to attempt to demonstrate their complete accordance with the theory.

Von Weimarn [1914] has shown that the form of the precipitate produced in a double decomposition of the type $AB + CD = AC + BD$ is dependent on the number, N, of crystallisation centres produced, determined by the formula:

$$N = \frac{P}{L} K_{AB} \cdot K_{CD} \cdot K_{BD} \cdot K_{AC} \cdot Z \ldots ,$$

where P is the mass of the substance AC to be deposited as precipitate,

L, the solubility of the precipitate,

K_{AB}, K_{CD}, etc., the degrees of aggregation, in solution, of the four components of the reaction, and

Z, the viscosity of the reaction medium.

The value of N is also proportional to the degree of aggregation of the reaction medium and of any other substance, or substances, present therein which do not enter into the double decomposition.

The occurrence of stratification in gels is therefore dependent on N, since it is only in cases where the precipitate is sufficiently finely divided that the adsorbing surface will be large enough to exhaust the solute from the adjacent region of gel. As the value of N decreases, in the presence of sufficient nutrient material, so the size of the individual aggregates, or crystals, will

increase, and the area of adsorbing surface diminish, until there is insufficient surface to produce stratification. On the contrary the greater the value of N, the more gelatinous the precipitate, and the slower the rate of diffusion of the precipitant through the layer of precipitate. So that, with very large values of N, the precipitant cannot permeate the precipitate in time to traverse the exhausted layer of gel immediately beyond, before this is replenished with solute by diffusion from the gel. Precipitation will then be continuous. When in this connection it is remembered that the solution in the gel must be sufficiently dilute to allow of its exhaustion from the region adjacent to the precipitate, giving a small value of P, it will be clear that zone formation can occur only in cases in which L is very small, and within somewhat narrow limits. This is fully confirmed by observation.

Since, however, the already dilute solutions meet in the gel only by diffusion, the value of P will be actually much smaller than that corresponding to the concentration of the hypotonic reagent. Consequently there will be a tendency for the formation of larger crystals than would result from the instantaneous mixing of aqueous solutions.

Under similar conditions Dreaper [1914] obtained large crystals of lead chloride and ferrocyanide by allowing the reacting solutions to diffuse in opposite directions through a capillary tube. Indeed the process of allowing the slow mixing of solutions has long been known as a method for the preparation of crystals of sparingly soluble substances.

The occurrence of such large crystals is a remarkable feature of reactions in gels. This is particularly noticeable in silicic acid gels, frequently observed in agar and more rarely in gelatin. It is thus evident that the gel medium has itself an extraordinary influence on the form of the precipitate resulting from a particular double decomposition, which tends to counteract, in a greater or less degree, the effect of the slow diffusion. Many examples of the influence of the gel medium on the value of N could be given, and it is convenient to consider this in connection with the effect of altering other physical conditions. The original Liesegang reaction with silver bichromate takes place readily in gelatin gels of various concentrations, while in 1 % agar remarkable ribbon-like crystals are formed which are much too large to cause stratification. If, however, the reagents are reversed, so that the silver salt is contained in the gel, beautiful laminae again result. These are, however, almost black and crystalline instead of red-brown and apparently amorphous as in gelatin. This may be attributed to an increased value of N resulting from the effect of a larger mass of that solute which is in the

state of greater aggregation, as in the case of the gelatinous precipitate of lead iodide, produced with basic lead acetate, mentioned below. If potassium chromate be substituted for the bichromate, only detached crystals are formed, without a trace of stratification; though here, of course, the crystals consist of silver chromate instead of bichromate. Returning to the case of the agar gel containing potassium bichromate, if the concentration of the solute be considerably diminished, the size of the crystals rapidly falls and an unmistakable tendency to stratification begins to appear, until, with a concentration of $N/100$ $K_2Cr_2O_7$, two separate bands of tiny rounded brownish black particles are formed at a distance of 2·5 mm. apart. With still smaller concentrations dark reddish brown colorations are noticeable in the weaker part of the gel below the bands, indicating sol formation. On reducing the solubility of the precipitate by mixing the agar gel with half its volume of absolute alcohol, an ordinary concentration of $N/20$ $K_2Cr_2O_7$ gives several bands of tiny feather-like crystals, 2 mm. thick, separated by clear spaces, with indications of sol formation in the lower part of the tube.

The case of lead iodide differs from that of silver bichromate in that beautiful laminations are produced in 1 % agar, and, as stated by Hatschek [1914], only a continuous gelatinous precipitate is formed in gelatin. The statement appears, however, to refer to the concentrations of the reagents used in 1 % agar, viz., 4 % of KI in the gel treated with concentrated $Pb(NO_3)_2$. The value of N in this case is too large. By using $N/20$ KI, and $N/1$ $Pb(NO_3)_2$, or $N/1$ $Pb(C_2H_3O_2)_2$, the precipitate becomes more permeable and stratification occurs in the lower half of the tube, although the precipitate is still much more gelatinous than in agar. A similar gelatinous precipitate is also obtained in agar by treating a gel containing $N/20$ KI with basic lead acetate solution. The number of crystallisation centres is increased by the greater aggregation of the precipitant and the precipitate becomes almost impermeable, so that only a few bands are produced. When the reaction takes place in silicic acid gel containing about 3·5 % of silica, which allows about the same rate of diffusion as in 5 % gelatin and 1 % agar, no stratification is visible, but the salt is deposited in large crystals and growth and dendritic forms. Von Weimarn has shown that growth forms and spherical crystals are conditioned by a value of N which is intermediate between those corresponding to complete crystals and so-called amorphous precipitates.

Lead chromate forms a gelatinous precipitate in agar so that it is only in very dilute concentrations, about $N/100$, that stratification occurs, the

result being then very beautiful. In gelatin the precipitate is too gelatinous to stratify, while in silicic acid crystalline needles are produced. Cupric phosphate gives a gelatinous precipitate in agar, which however becomes distinctly stratified in very dilute concentrations, whereas in silicic acid it yields remarkably beautiful deep blue discs. The basic hydroxide also gives good bands in silicic acid, but has not been obtained, so far, other than as a continuous gelatinous precipitate in test tubes containing agar gel. However, by working with U-tubes, half filled with pure agar gel between the reacting solutions in the limbs above, the value of P is so much reduced where the liquids meet that stratification takes place even with an initial concentration of $N/20$. Zone formation is assisted in this case by the increased facility with which the gel can be exhausted of solute owing to the great dilution in the neighbourhood of the meeting solutions.

From these considerations it will be clear that the formation of a banded precipitate is dependent on the number of crystallisation centres produced, and that this quantity is affected by the properties of the gel in which the reaction takes place. This influence of the reaction medium may be explained by the fact that it is actually a dilute aqueous solution of the gel substance in a high degree of aggregation. The number of crystallisation centres is therefore proportionally increased. Under conditions of sufficient dilution the production of sols would be favoured.

The well-known "protective values" of gelatin, agar and silicic acid are in the proportion which would explain the observed variations in the form of the precipitate. In the masterly paper previously quoted, in which he collects together the great variety of facts which require explanation, Hatschek suggests that this protective action may eventually be found to be connected with the behaviour of different gels.

Sol formation is however unnecessary for the production of a stratified precipitate, and could hardly occur, except momentarily, in normal cases. Dreaper [1914] obtained well-defined bands of copper sulphide in columns of sand, which has no protective action. Although in gelatin, particularly, the instantaneous formation of a sol is possible on the first meeting of the diffusing solutions, it would be coagulated immediately by the rapid increase in the value of P leading merely to a more gelatinous precipitate; and it is noticeable that in cases where sol formation is most probable it is usually most difficult to induce stratification. As Hatschek has clearly demonstrated, supersaturation, in any form, is not adequate to explain the many varied cases of zone formation. Nor does it afford an explanation of the very different

results obtained when the solutions in and above the gel are interchanged, as for example with lead iodide, as previously recorded [Bradford, 1916].

The microscopic observation of reactions in gels has already been carried out by Liesegang [1913] for silver bichromate, and by Hatschek [1914] for lead iodide and chromate. The experiments were made in the plate form. The original descriptions are here reproduced. "On observing the region beyond the last line, for some time one perceives nothing, although, as remarked, silver chromate must be forming. Suddenly a new line appears at a distance of half a millimetre. In distinction from the previous line, which appears dark red by reflected light and black by transmitted light, the new line is yellow and very tenuous. In the course of the next ten seconds it becomes considerably intensified and fiery red in colour, but does not attain the density of the older line for some time. This solidification, which is coupled with a darkening of the colour, is reached in about three minutes." It is convenient to consider here a point raised by Hatschek [1914], in connection with the same reaction. "The drop of silver nitrate diffuses into the bichromated gelatin producing, at first, a band of silver bichromate two or three millimetres wide before ring formation commences, and, even if the concentration of silver nitrate be reduced to 5 %, a similar wide band is still built up." According to the adsorption theory, the silver nitrate diffuses into the gel containing potassium bichromate, forming a ring of precipitate of silver bichromate. This precipitate immediately begins to adsorb solute from the gel, which is precipitated as it collects, with constantly growing surface and increasing adsorbing power. In this way the region of gel next the precipitate tends to become gradually exhausted of potassium bichromate and fresh material diffuses towards this region from the more distant parts of the gel. A concentration gradient is thus set up towards the zone of gel in contact with the adsorbing precipitate, and this region eventually becomes so dilute that no further precipitate is formed. The wide band then ceases to grow. It is evident, therefore, that the formation of this wide band depends on the concentration of the hypotonic reagent in the gel, rather than on that of the precipitant. In the case of U-tube experiments this concentration gradient is already in existence when the diffusing solutions meet, and these are so dilute that band formation generally commences immediately. Considering now Liesegang's description: the silver nitrate slowly diffuses through the previous rings of precipitate into a region from which the potassium bichromate has been adsorbed, and no further silver bichromate is formed (not even as a sol, since this would

be indicated by its colour[1]), until the precipitant meets the bichromate diffusing in from the gel. A faint new ring is then generated. The new precipitate commences to adsorb fresh solute from the gel, which is precipitated as it collects. The density of the ring, and incidentally the adsorbing surface, increases. As the solute is rapidly adsorbed from the thin layer of gel immediately in contact with the precipitate, fresh solute slowly diffuses towards this region, until the layer of gel adjacent to the precipitate again becomes exhausted of solute. The new ring will then cease to grow, and the whole process will be repeated until the available solute in the gel has been precipitated.

Since the adsorbed solute collects on the surfaces of the freshly deposited particles of the precipitate, the value of P/L will be greater there than elsewhere. Consequently precipitation will take place on the surfaces of the grains of the precipitate, which will grow equally in all directions. A further increase in concentration may result in the formation of an additional number of smaller centres, but the individual particles will grow in the same way. Microscopic examination of the rings and bands invariably reveals the spherical or approximately spherical shape of the constituent grains of the precipitate, which frequently reach macroscopic size. In many cases larger particles are seen imbedded in a finer grained precipitate. Hatschek's description of the lead iodide reaction records the observation of the actual growth of such spherical aggregates, which are characteristic of reactions in gels. "Here also the reaction begins with the appearance of a light yellow coloured strip. However, at the same time, numerous points of lead iodide appear on the inner side of the band which grow remarkably while the strip is only increasing slightly in intensity and width. When the isolated grains seem to have reached their full size, the band appears to grow suddenly and become opaque, but it has an essentially coarser structure than the silver chromate ring."

The progress of the lead chromate reaction is exactly the same as that of the lead iodide. The coarser structure of the bands is due to a smaller value of P/L, and allows the precipitant to diffuse through more quickly than in the case of silver bichromate. This has the same effect as if the diffusion gradient of the solute in the gel were less. The precipitant enters a region where the resulting value of P/L is very small, and comparatively few crystallisation

[1] Cases of sol formation which have been observed have always occurred at the end of the reaction, after the solvent has been almost completely extracted from the gel. The appearance is then merely of a diffuse coloration without any indication of stratification.

centres are produced. The excess of precipitant, flowing on, meets with constantly increasing quantities of solute, N grows proportionally, until the precipitated particles appear to form a band, and as their number increases, so their size diminishes. This corresponds exactly to the microscopic appearances, and may be taken as a further direct proof of the existence of a concentration gradient. The isolated particles appear simultaneously with the ring, because it is not until they have reached a certain size that they become separately visible. Actually they may be formed somewhat earlier. They grow by adsorption of nutrient material from the adjacent gel, until this is exhausted, and thickening of the ring cuts off further supplies. By this time the region of the new band has increased in concentration of both the inflowing reagents, resulting, at first, in a denser precipitate and a quicker rate of adsorption and precipitation, until, in consequence of the adsorption, the concentration of the adjacent gel falls once more.

One other point must be mentioned in conclusion. It has been insisted that stratification is a rare phenomenon limited to a few insoluble precipitates. This is not in accordance with the writer's experience. Although experiments have been directed to the examination of the conditions underlying zone formation in a limited number of cases, rather than to the multiplication of specimens, successful attempts have been made to prepare stratified deposits of those substances such as silver chloride, barium sulphate, copper phosphate in agar, and lead iodide in gelatin which have been specifically mentioned as refusing to stratify. No serious attempt to obtain a sufficiently insoluble precipitate in the stratified form has failed, except in the case of aluminium hydroxide in agar in U-tubes, and this was to be expected on account of its extreme insolubility. There appears to be no evidence to show that stratification will not occur provided a precipitate can be obtained in a suitable state of subdivision from solutions of a sufficient dilution: or that the production of such precipitate is other than a question of the influence of the various known physical factors. As opportunity offers, it is hoped, by increasing or diminishing the number of crystallisation centres in various ways, to prepare well stratified precipitates of substances in gels in which they are not ordinarily so obtained.

REFERENCES.

Bradford (1916), *Biochem. J.* **10**, 169.
Dreaper (1914), *Kolloid Zeitsch.* **14**, 163.
Hatschek (1914), *Kolloid Zeitsch.* **14**, 115.
Liesegang (1913), *Geologische Diffusionen*, p. 89.
v. Weimarn (1914), *Zur Lehre von den Zuständen der Materie.*

V. THE REACTION OF SERA AS A FACTOR IN THE SUCCESSFUL CONCENTRATION OF ANTITOXIC SERA BY THE METHODS AT PRESENT IN USE.

By ANNIE HOMER.

From the Lister Institute, Elstree.

(*Received February 23rd, 1917.*)

In a previous communication [1916, 1] I suggested that the practical difficulties so often encountered in the filtration of concentrated antitoxic sera might be due to the fact that hitherto no cognisance had been taken of the reaction of the sera before being concentrated.

With this object in view experiments have been carried out in which the effect of variations in the reactions of the sera have been considered with reference to:

(i) The precipitation of serum proteins by 30 per cent. of saturation with ammonium sulphate.

(ii) The increased precipitation of the serum proteins during the shortened heating process in the Banzhaf (1913) method. [Banzhaf, 1913.]

(iii) The increased precipitability of the serum proteins during the preliminary heating of the plasma as suggested in a previous paper. [Homer, 1916, 2.]

(iv) The practical application of the results obtained from the above investigations.

During the investigation of these points there has been accumulated a considerable amount of data which shows that the reaction of the serum is an important factor in the successful precipitation and heat denaturation of the serum proteins and that, unless the reaction be in some way controlled, apparently inexplicable irregularities are apt to occur in the course of the concentration.

I. The effect of changes in the reaction of serum on the precipitation of the serum proteins by 30 per cent. of saturation with ammonium sulphate.

Several series of experiments were conducted with oxalated plasma from different horses and from batches of pooled plasma or serum made up for concentration.

In each series, separate volumes of the test plasma were treated with known volumes of acid and of alkali. The plasma, thus treated, was brought up to 30 per cent. of saturation with ammonium sulphate by the addition of the necessary volume of a saturated solution of ammonium sulphate. The hydrogen ion concentration of the plasma and of the plasma-ammonium-sulphate mixtures was measured, wherever possible, by means of indicators and Sörensen's solutions in Walpole's colorimeter. The determinations, thus made, were subsequently checked by the electrical method as it was found that many of the indicators were rendered unreliable in the presence of so much protein as there is in serum. The protein contents of the filtrates from the serum mixtures were measured by means of the Zeiss immersion refractometer.

TABLE I.

Showing the influence of changes in the hydrogen ion concentration of the serum-ammonium-sulphate mixtures on the precipitation of serum proteins by 30 % *of saturation with ammonium sulphate.* (*Protein content of the plasma* = 7·92 %. *Hydrogen ion concentration of the saturated solution of ammonium sulphate added to the plasma* = p_H^+ 6·480.)

Volume of normal acid (or equivalent) added to 100 cc. of the diluted plasma	Volume of normal alkali (or equivalent) added to 100 cc. of the diluted plasma	p_H^+ of the diluted plasma to which the acid or alkali has been added, measured by the electrical method	p_H^+ of the filtrate from the serum-ammonium-sulphate mixtures, measured by the electrical method	Residual percentage of protein in solution in the filtrates from the serum-ammonium-sulphate mixtures, measured by means of the refractometer
—	19·6 cc. N . NH_3	—	8·754	5·75
—	13·1 ,,	—	8·551	5·80
—	6·55 ,,	9·945	8·455	5·90
—	3·28 ,,	9·789	8·022	6·00
—	1·96 ,,	9·304	7·745	6·00
—	0·98 ,,	8·500	7·400	6·30
0·00 cc. N . HAc	0·00 ,,	7·430	7·077	6·30
0·38 ,,	—	—	7·000	6·52
1·25 ,,	—	6·450	6·438	6·60
2·80 ,,	—	5·921	6·021	6·60
3·60 ,,	—	5·440	5·579	6·25
3·76 ,,	—	5·225	5·327	6·20
3·99 ,,	—	5·025	5·145	5·87
4·99 ,,	—	4·600	4·972	5·02
6·00 ,,	—	4·560	4·712	4·52

The data with respect to one of the series of experiments have been embodied in Table I; comparable results were obtained with all the samples of serum and plasma investigated.

A study of Table I shows that within certain limits (p_H^+ 5·3 to 7·4) the reaction of the mixtures has little effect on the extent of the precipitation of the serum proteins. Beyond these limits in both directions there is an increased precipitation of protein by the sulphate. It was also noticed that the consistency of the precipitated euglobulin was greatly influenced by the reaction of the mixtures. The agglutination of the precipitate into a granular and readily filterable form proceeded most satisfactorily at a hydrogen ion concentration of about p_H^+ 5·3 to 5·0, i.e. on the slightly acid side of the isoelectric point of euglobulin (p_H^+ 5·5).

The state of aggregation of the Second Fraction precipitate (50 % of saturation with ammonium sulphate) was also dependent on the reaction of the mixture: the conditions most favourable for this precipitation are in the course of investigation.

II. The effect of changes in the reaction of the serum on the increased precipitation of the serum proteins induced during the shortened heating process in Banzhaf's (1913) method.

Several series of experiments were undertaken with samples of oxalated plasma from individual horses and from batches of pooled plasma from different horses.

In each series, to separate volumes of the test plasma contained in stoppered bottles, was added a known amount of acid or of alkali together with the volume of a saturated solution of ammonium sulphate necessary to bring the sulphate content to 30 % of saturation. The hydrogen ion concentration and the protein content of the clear filtrates from each of the experimental liquids were measured in the usual way. Experiments were carried out both with undiluted plasma and with plasma diluted with one-third its volume of water.

The mixtures were heated and then filtered. The protein content of the filtrates was estimated and compared with the corresponding value for the unheated liquids. From the data thus obtained was calculated the percentage increased precipitation of the soluble proteins due to the heating of the mixtures.

Comparable results have been obtained with each of the sera examined

irrespective of their protein content and of their dilution with water. The data with respect to one of the series of experiments have been embodied in Table II and represented graphically in the curve in Fig. 1.

TABLE II.

Showing the influence of changes in the reaction of the sera on the increased precipitation of protein during the heating of the serum-ammonium-sulphate mixtures to a temperature of 61° and keeping them at that temperature for 7 to 10 minutes. (Protein content of the plasma = 8·72 %. The saturated solution of ammonium sulphate added to the plasma had a hydrogen ion concentration p_H^+ 6·480.)

Volume of normal acid (or equivalent) added to 100 cc. of plasma diluted with one-third its volume of water	Volume of normal acid (or equivalent) added to 100 cc. of the diluted plasma	p_H^+ of the serum-ammonium-sulphate mixtures determined by the electrical method	Percentage increase in the precipitation of the soluble proteins induced during the heating of the serum-ammonium-sulphate mixtures	Remarks on the filtration of the heated serum-ammonium-sulphate mixtures
—	19·64 cc. N . NH_3	8·754	17·7	Readily, filtrate clear
—	13·04 ,,	8·577	16·4	,, ,, ,,
—	6·52 ,,	8·455	13·4	,, ,, ,,
—	3·28 ,,	8·022	10·0	,, ,, ,,
—	1·96 ,,	7·745	7·0	,, ,, very slightly opalescent
—	0·98 ,,	7·489	9·0	,, ,, ,, ,,
—	0·49 ,,	7·312	7·0	Less readily, filtrate slightly opalescent
0·00 cc. N . HAc	0·00 ,,	7·077	6·3	,, ,, ,, ,,
0·98 ,,	—	7·000	6·8	Slowly, filtrate opalescent
1·96 ,,	—	6·428	3·0	,, ,, ,,
2·94 ,,	—	6·021	2·8	,, ,, ,,
3·60 ,,	—	5·579	0·5	,, ,, ,,
3·76 ,,	—	5·327	18·0	Less slowly, filtrate slightly opalescent
3·92 ,,	—	5·145	20·7	Readily, filtrate clear
4·58 ,,	—	5·001	39·0	,, ,, ,,
4·88 ,,	—	4·972	52·6	,, ,, ,,
5·88 ,,	—	4·712	72·2	,, ,, ,,

It was noticed that where the hydrogen ion concentration of the mixtures was between the values p_H^+ 5·3 and 7·0, the filtration of the heated liquids was slow and the filtrates were markedly opalescent. Throughout these ranges the conversion of soluble into insoluble protein was least. In those cases in which the reaction had been more acid or more alkaline than in the above the filtration of the mixtures was rapid and the filtrates were clear, while the conversion of soluble into insoluble protein was of an appreciable order. On the alkaline side between the ranges p_H^+ 7·0 and 8·7 the conversion gradually rose from 5 to 17 %. On the acid side, as is seen at a glance in the

curve in Fig. 1, the rate of increase was much more marked; it rose from 0·5 to 72 % between the ranges p_H^+ 5·5 and 4·7.

No trouble was experienced with the filtration of the final products obtained in the concentrations of sera in which the reaction of the serum mixtures previous to heating had been adjusted to p_H^+ 5·2 or p_H^+ 5·0. As was however to be anticipated from the character of the filtration of the hot serum mixtures, difficulties were encountered with the end products where the reaction of the serum mixtures had been between the limits p_H^+ 5·3 and 7·0. In these cases the unsatisfactory filtration of the serum mixtures may be ascribed to one of two possible causes; either that between these limits the

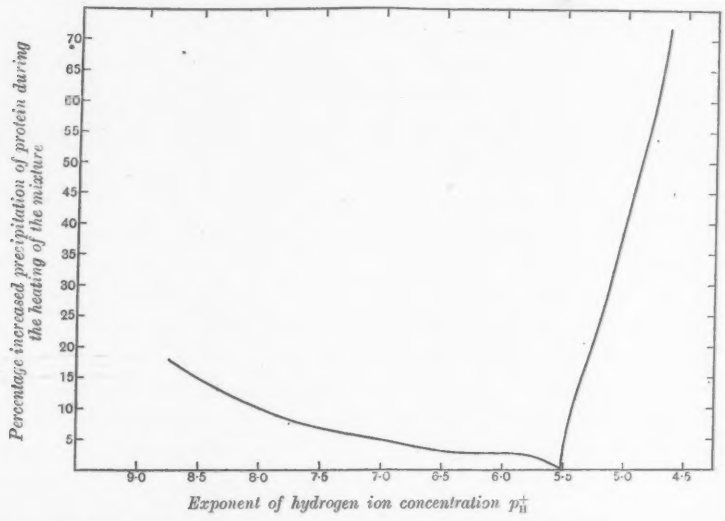


Fig. 1. Curve representing the influence of the reaction of the serum mixtures on the percentage increased precipitation of soluble proteins during the heating of serum-ammonium-sulphate mixtures by Banzhaf's method.

hydrogen ion concentration is unfavourable for the complete agglutination of the precipitated proteins: or that, since near the isoelectric point the heat denaturation of the serum proteins is least, it is possible that in this region the proteins which, under more acid or more alkaline conditions, would be appreciably denaturated are in a transitory, semi-emulsoid, semi-rigid stage and as such impart a slimy consistency not only to the serum mixtures but also to the final products.

It was also found that, with serum mixtures more alkaline than p_H^+ 8·0, there was difficulty with the filtration of the final product even though the filtrates from the heated serum mixtures were clear. In these cases, when these clear alkaline filtrates were acidified to a point p_H^+ 5·5 an opalescence was produced. The presence of a certain amount of precipitated protein which had been kept in the disperse phase by the alkali was thus indicated. The colloidal aggregates thus dispersed escaped precipitation with the First Fraction precipitate but were subsequently precipitated with the Second Fraction precipitate. They impart to the residue from the dialysis of the latter a slimy consistency and thereby considerably impede filtration through filter candles.

III. THE EFFECT OF CHANGES IN THE REACTION OF SERA ON THE INCREASED PRECIPITABILITY OF THE SERUM PROTEINS INDUCED DURING THE HEATING OF SERUM TO A TEMPERATURE OF 57° FOR 6 HOURS.

During the course of our practical experience with the method suggested by me [Homer, 1916, 2] for the concentration of antitoxic sera it was observed that the heat denaturation of the serum proteins induced during the preliminary heating of the sera varied from 13 to 45 %. Throughout the winter and spring months the values with plasma varied from 28 to 35 % while with carbolised sera values as high as 45 % were reached. During the summer months when the horses were being given a considerable amount of green grass, the denaturation varied from 13 to 20 %.

After a considerable amount of experimental work it was ascertained that these variations were due to differences in the hydrogen ion concentration of the different plasmas and sera. During the summer months, when the low values for the heat denaturation were obtained, the oxalated plasma was slightly less alkaline than that obtained during the winter and spring months.

Samples of plasma from individual horses and from batches of the pooled plasmas from different horses were respectively diluted with one-third their volume of water. The hydrogen ion concentration of the diluted plasma was measured in each case. The reaction of the various samples was adjusted to the value of about p_H^+ 5·6 to 5·9 and also to the value p_H^+ 8·5. The original samples and the adjusted samples were heated in stoppered bottles to a temperature of 57° for 6 hours. The percentage increased precipitability of the serum proteins by 30 % of ammonium sulphate was measured with the aid of the Zeiss refractometer.

The data with respect to these observations have been embodied in Table III. They show that the variations in the extent of the heat denaturation of the serum proteins of individual sera can be obviated by adjusting the reaction of the sera to some fixed value. Thus, when the reaction was adjusted to about p_H^+ 5·6 to 5·9 the denaturation was of the order of 10 %, while at the value p_H^+ 8·5 it was of the order of 40 %[1].

TABLE III.

Showing that the variation in the heat denaturation induced during the heating of different samples of plasma at 57° for 6 hours can be overcome by an adjustment of the reaction of the sera to a fixed degree of acidity or of alkalinity.

Sample No.	Percentage protein content of the plasma	Value for p_H^+ of the diluted plasma	Percentage increased precipitability of the serum proteins during the heating of: undiluted plasma	diluted plasma brought, by the addition of ammonia, to the hydrogen ion concentration p_H^+ 8·5*	diluted plasma brought, by the addition of acetic acid, to the hydrogen ion concentration p_H^+ 5·9*
1	8·59	7·648	31·1	—	—
2	8·12	7·503	28·9	44·0	13·0
3	7·80	7·460	26·4	41·0	9·0
4	8·59	7·563	28·4	40·0	12·0
5	6·56	7·430	22·3	41·0	13·0
6	7·57	7·346	23·5	40·5	—
7	7·92	7·346	22·0	—	8·7
8	6·55	7·863	39·0	43·0	13·0
9	6·62	7·346	21·0	41·0	9·0
10	6·60	7·346	23·0	42·0	—
11	8·40	6·813	13·5	40·5	—
12	10·03	6·990	13·5	40·5	—
13	7·15	7·000	17·0	40·0	—

[Similar results were obtained with undiluted plasma.]

* Measured by means of indicators and Walpole's colorimeter.

In order to elaborate the point still further experiments were instituted in which samples of different sera, previous to their being heated, were treated with acid and with alkali to ensure a gradation in the reaction of the sera from p_H^+ 4·2 to 9·9.

Comparable results were obtained with each series of experiments. The typical results from one of these series have been embodied in Table IV and

[1] These adjustments were made with the aid of indicators and Sörensen's solutions in Walpole's colorimeter. A certain amount of difficulty was experienced by me in the matching of colours in serum, especially so, with the indicators available, on the acid side. I subsequently ascertained that, owing to my defective matching, the real values of the hydrogen ion concentration varied from the values aimed at by amounts sufficient to account for the slight variations in the heat denaturation recorded in the Table.

TABLE IV.

Showing the influence of the hydrogen ion concentration of sera on the extent of the heat denaturation of the serum proteins induced during the prolonged heating of serum at a temperature of 57° for 6 hours.

Volume of normal acid (or equivalent) added to 100 cc. of the plasma diluted with one-third its volume of water	Volume of normal alkali (or equivalent) added to 100 cc. of the plasma diluted with one-third its volume of water	p_H^+ of the plasma determined by the electrical method	Appearance of the heated plasma	Percentage conversion of soluble into insoluble protein during the heating of the diluted plasma A	Percentage increase in the precipitability of the soluble proteins by 30% of saturation with ammonium sulphate B	Percentage destruction of antitoxin during the heating process
—	6·55 cc. N . NH_3	9·945	clear	2·2	55·7	nil
—	4·92 ,,	9·910	,,	2·0	53·5	
—	3·275 ,,	9·789	,,	1·5	46·6	
—	2·62 ,,	9·477	,,	1·5	45·5	
—	1·96 ,,	9·304	,,	2·2	46·5	nil
—	1·31 ,,	8·870	,,	3·7	41·5	
—	0·65 ,,	8·249	clear liquid and slight deposit	2·2	36·8	
0·00 cc. N . HAc	0·00 ,,	7·430	,, ,, ,, ,,	3·3	22·3	nil
0·63 ,,	—	6·990	opalescent liquid and filtrate	5·2	10·2	
1·25 ,,	—	6·450	,, ,, ,, ,,	5·2	12·0	nil
1·87 ,,	—	5·921	,, ,, ,, ,,	3·7	13·1	
2·49 ,,	—	5·440	thin suspension, clear filtrate	6·3	13·1	
3·74 ,,	—	5·225	,, ,, ,, ,,	20·8	25·7	nil
2·50 cc. N . H_2SO_4	—	5·160	,, ,, ,, ,,	26·0	35·8	nil
4·17 cc. N . HAc	—	5·025	thick ,, ,, ,,	30·0	45·0	
2·52 cc. N . HCl	—	5·000	,, ,, ,, ,,	34·0	44·2	
4·99 cc. N . HAc	—	4·600	,, ,, ,, ,,	54·0	70·0	nil
3·60 cc. N . HCl	—	4·635	,, ,, ,, ,,	54·0	74·0	
3·60 cc. N . H_2SO_4	—	4·643	,, ,, ,, ,,	50·0	74·0	
6·74 cc. N . HAc	—	4·560	,, ,, ,, ,,	68·0	77·5	nil
4·40 cc. N . H_2SO_4	—	4·262	almost solid	98·0	not fluid enough to be measured	
4·36 cc. N . HCl	—	4·251	,, ,,	92·0		

With the sera ranging from p_H^+ 9·9 to 5·4 the extent of the changes recorded in *B* was a function of the time of heating, whereas with the more acid sera the change was practically completed within one hour's heating at the specified temperature.

have also been represented graphically in the curves in Fig. 2. Of the two, Curve 1 illustrates the changes in the solubility of the serum proteins during the heating of the serum, while Curve 2 indicates the accompanying changes in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate.

It was found that, with each of the sera examined, the extent of the changes involved was a function of the hydrogen ion concentration of the serum irrespective of the protein content: the use of undiluted serum or plasma gave rise to results similar to those obtained from experiments with plasma diluted with one-third its volume of water.

The appearance of the heated sera was characteristic throughout the ranges of acidity and alkalinity investigated.

It was found that the heated sera were clear when the reaction of the unheated serum had been more alkaline than p_H^+ 8·3. When the reaction had been between the limits p_H^+ 8·2 and 5·5 the heated sera were characterised by an opalescent appearance or by the presence of a slight deposit of protein. On the acid side of p_H^+ 5·5 the sera were converted, during the heating, into thin suspensions, the semi-solid consistency of which became more marked as the degree of acidity increased, until at p_H^+ 4·2 the serum became practically solid.

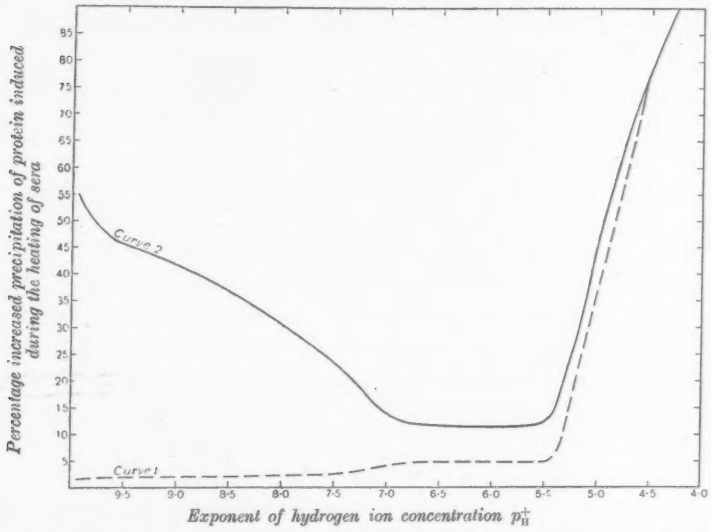


Fig. 2. The curve — — — represents the influence of changes in the reaction of sera on the percentage conversion of soluble into insoluble proteins induced during the heating of sera at 57° for 6 hours.

The curve ———— represents the influence of changes in the reaction of sera on the percentage increased precipitability of the serum proteins by 30 % of saturation with ammonium sulphate induced during the heating of sera at 57° for 6 hours.

From a study of the data given in Table IV, Column A, it will be seen that the conversion of soluble into insoluble protein was of the order of 5 % or less in the sera of which the reaction lay between the ranges p_H^+ 9·9 and 5·5. On the acid side of p_H^+ 5·5 there was marked precipitation of the soluble proteins; thus at a hydrogen ion concentration of p_H^+ 5·4 it was of the order of 6 %, at p_H^+ 5·0 it was 30 %, while at p_H^+ 4·2 it was over 90 %.

From a consideration of Column B in the same table it will be seen that, irrespective of any accompanying conversion of soluble into insoluble protein, there was throughout the whole range investigated a marked increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate: the extent of the increase was a function of the reaction of the serum previous to its being heated.

This increased precipitability was least between the ranges p_H^+ 5·4 and 7·0. On either side of these limits the rate of increase became apparent: the gradient for the increase was more gradual on the alkaline than on the acid side (Curve 2, Fig. 2). As the degree of alkalinity decreased from p_H^+ 9·9 to 8·0 the increased precipitability gradually fell from 45 to 30 %. Between the ranges p_H^+ 7·0 and 5·5 it was of the order of 10 to 15 %; on the acid side it rapidly rose from 25 % at p_H^+ 5·2 to 90 % at p_H^+ 4·2.

It was also interesting to note that, in all cases, there was no appreciable loss of antitoxin during the heating of sera the reaction of which had been adjusted to degrees of acidity or alkalinity within the limits dealt with in the accompanying tables, although under some conditions the extraction of the antitoxin from the heat-denaturated proteins proved a matter of difficulty.

From a study of the curves in Fig. 2 it is apparent that while on the alkaline side the configuration of Curve 1 is quite distinct from that of Curve 2, on the acid side of p_H^+ 5·5 there is practical coincidence. There is, moreover, a marked similarity between the configuration of Curve 2 and that of the curve in Fig. 1. The extent of the increased precipitability on the alkaline side is not so marked in the latter as in the former, but on the acid side the curves, if superimposed, would be almost identical.

The question then arose as to whether the individuality of Curves 1 and 2 (Fig. 2) on the alkaline side indicated some radical difference between the changes respectively involved during the heating of alkaline and acid sera.

In order to elucidate this point samples of the clear heated alkaline sera which showed a 40 % increased precipitability by the sulphate were brought to an acidity of p_H^+ 5·0. Now, had the changes involved during the heating of the alkaline sera been the same as those induced in the acid sera which showed a corresponding percentage increased precipitability, acidification of the clear alkaline sera should have changed the consistency of the latter to that of a thin suspension (*vide ante*, p. 29). A slight opalescence only was formed and the refractometer readings demonstrated that the quantity of protein thus precipitated was inappreciable. The opalescence therefore

probably indicated that a small amount of heat changed protein had been kept in the disperse phase by the excess of alkali (*vide ante*, p. 26).

It was also noticed that the increased precipitability of the proteins of the heated alkaline sera was a function of the duration of the heating process. On the other hand the changes recorded in the acid sera were practically complete after one hour's heating at the specified temperature.

From these observations it is evident that the changes taking place during the heating of alkaline sera are of a different nature from those which occur during the heating on the acid side of the isoelectric point of the globulins. For this reason it is possible that if the heat denaturation be conducted on the acid side, there may be prepared concentrated products which will be less likely to give rise to the troubles of anaphylaxis and of serum sickness than the present products concentrated by methods involving a denaturation of the serum proteins by heating the slightly alkaline sera.

Practical application of the results obtained in Sections I, II and III above.

The results of the investigation detailed above have led us to a better understanding of the causes underlying the irregularities encountered in the concentration of sera by methods involving the heating of the serum previous to the fractional precipitation of the serum proteins by ammonium sulphate.

We are finding that the production of clear and readily filterable end products can only be assured by controlling the reaction of the serum and of the serum mixtures unless certain expedients described below be resorted to.

An account of the investigation undertaken to overcome the causes of irregularities in the results obtained in (A) the Banzhaf (1913) method and later in (B) the method suggested by me [1916, 2] may be of service to those engaged in the concentration of antitoxic sera.

(A) *The regulation of the factors causing irregular results in the concentration of sera by the Banzhaf* (1913) *method.*

It seems probable that the difficulties in filtration so often encountered in this method are due to the presence of incompletely precipitated euglobulin and also of the small amount of protein denaturated during the heating of the serum.

As an outcome of the work recorded above it has been concluded that,

with the published technique, there can be no guarantee for the complete elimination of these disturbing factors, and this for two reasons:

(1) That no cognisance has been taken of the reaction of the serum-ammonium-sulphate mixtures previous to their being heated.

(2) That there is no increased precipitation of the serum proteins during the heating of the serum mixtures.

It is generally recognised that rapid and clear filtration of the hot serum mixtures is one of the essential conditions for the production of readily filterable end products. In this laboratory it has been shown that, for the successful filtration of the hot serum mixtures, there must be assured the agglutination of the particles of precipitated euglobulin into aggregates sufficiently large for their retention on the surface of filter paper. From Tables I and II it is obvious that the success of this operation can only be guaranteed if the reaction of the mixtures be suitably adjusted.

It has been demonstrated that during the heating process advocated in this method the precipitating power of 30 % of saturation with ammonium sulphate is not appreciably enhanced. It is evident, therefore, that the small amount of euglobulin which must always escape precipitation with the First Fraction precipitate will appear in colloidal suspension in the final product. Unless the latter be allowed to stand for a considerable time until this colloidal suspension has settled as a deposit the unprecipitated protein proves a hindrance to filtration and is the cause of the marked clouding of so much of the antitoxic serum concentrated by this method.

The irregularities in the filtration of the serum mixtures can be obviated by an adjustment of the hydrogen ion concentration to the optimum required for the complete agglutination of the particles of "salted out" euglobulin and of the particles of protein denaturated during the heating of the serum. Good results can be obtained by adjusting the reaction to an acidity of about p_{H}^{+} 5·0. As however the determination of the hydrogen ion concentration of the sera by the electrical method is not always practicable in a routine laboratory, an endeavour is being made to work out a simple practical method by which the reaction can be adjusted by the use of suitable indicators.

In the meantime, pending the search for suitable and reliable indicators, it has been shown that the irregularities in the filtration of the serum mixtures can also be obviated by the simple expedient of adding 1·5 to 2 % of sodium chloride to the mixtures previous to their being heated.

It is important to know whether the salt improves the filtration of the hot serum mixtures by virtue of a specific action on the particles of precipi-

tated protein or by an adjustment of the reaction of the medium more nearly to the desired optimum.

In order to elucidate this point duplicate samples of several plasmas were taken. To one of each of the duplicate pairs was added 1·5 to 2 % of sodium chloride. The hydrogen ion concentration of the duplicates was measured. The liquids were then made 30 % of saturation with ammonium sulphate and gradually heated to 61°.

From the results given in Table V it will be seen that the addition of sodium chloride decreases the alkalinity of the serum and of the serum

TABLE V.

Showing the influence of the addition of sodium chloride to the serum on the hydrogen ion concentration of the serum and of the serum-ammonium-sulphate mixtures.

p_H^+ of the plasma diluted with one-third its volume of water = A	p_H^+ of A made 30 % of saturation with ammonium sulphate	Percentage of NaCl added to the diluted plasma	p_H^+ of the salted plasma = B	p_H^+ of B made 30 % of saturation with ammonium sulphate	Remarks on the filtrate from the hot serum-ammonium-sulphate mixtures from	
					A	B
7·485	—	1·5	7·216	—	opalescent	clear
7·408	—	2	7·200	—	,,	,,
7·701	—	1·5	7·485	—	slightly opalescent	,,
7·701	—	4	7·485	—	,, ,,	,,
7·701	—	8	7·485	—	,, ,,	,,
7·347	—	2	7·223	—	opalescent	,,
—	7·250	1·5	—	7·010	,,	,,
—	7·220	2	—	7·000	,,	,,

mixtures. From a comparison with the data given in Table II, it is however obvious that the change in the hydrogen ion concentration induced by the addition of the salt is insufficient to account for the marked improvement in filtration and for the absence of an opalescent suspension in the filtrates.

It seems justifiable, therefore, to conclude that the improvement in filtration is induced by a specific action of the sodium chloride on the particles of precipitated protein whereby their satisfactory agglutination is assured.

Although the salt greatly improves the filtration of the serum mixtures, it cannot completely obviate the difficulties with the final product for it is powerless to ensure the complete precipitation of the euglobulin by 30 % of saturation with ammonium sulphate. Even in the presence of salt the desired increased precipitation of the serum proteins during the heating process does not take place.

In order to guarantee the elimination of euglobulin from the final products there were three courses open, viz.

(i) *The old method of extraction with brine.*

The Second Fraction precipitate containing the pseudo-globulin and antitoxin together with the small amount of attendant euglobulin and heat-denaturated protein was thrown into brine. The pseudo-globulin-antitoxin combination passed into solution while the euglobulin remained undissolved and was separated by filtration. The pseudo-globulin and antitoxin were precipitated by the addition of 0·25 % of glacial acetic acid to the clear filtrate. The precipitate was pressed and dialysed; the end product was crystal clear and its filtration presented no difficulty as it contained no trace of euglobulin or of heat-denaturated protein.

From the practical standpoint this method is not favoured as it involves a considerable amount of extra time and labour.

(ii) *A combination of the prolonged heating process of the Gibson-Banzhaf method with the shortened heating process of the Banzhaf* (1913) *method.*

By the adoption of this course it has been possible to obtain end products which, while showing an increased potency of from 8 to 10 times that of the original serum, do not exhibit the same tendency to form the cloudy deposit so often seen in the sera concentrated by Banzhaf's method. However, recent observations on the factors limiting the extent of the concentration of sera by the fractional precipitation methods at present in use [Homer, 1917], have demonstrated that there is a limit to which the concentration of sera can be carried. It was found that in all cases where the load of antitoxin attached to one gram of protein in the final product threatened to be greater than of the order of 25,000 units, the excess of units beyond this limit were transferred to the First Fraction precipitate.

From these observations it is evident that, for high grade sera (over 600 units per cc.), the above modification, yielding under ordinary circumstances a concentration of 8 to 10 times, cannot be used without apparent loss of antitoxin. The missing antitoxin can be recovered by extraction of the First Fraction precipitate with saturated salt solution.

To provide for such cases a third method of procedure has been employed, viz.

(iii) *The addition of substances such as phenol and its homologues to the serum mixtures previous to their being heated.*

Some years ago MacConkey [1914] observed that, during the heating of carbolised and of etherised sera there was a considerable loss of antitoxin. He subsequently found that the loss was negligible if 1·5 to 2 % of salt had previously been added to the serum.

In this laboratory it has also been demonstrated that, in the presence of salt, there is usually no appreciable loss of antitoxin during the concentration of carbolised sera by Banzhaf's method, if the temperature of the serum mixtures be not raised beyond 61°. At temperatures of 63° and 65° the loss becomes appreciable.

In the routine work, however, it has sometimes happened that, during the concentration of carbolised sera, there were appreciable losses of antitoxin even in the presence of salt. The records showed that in some of these cases the serum had been heated to a temperature of 65°, a procedure which in itself was sufficient to account for the loss. In other cases the records showed that during the concentration there had been removed more than 80 % of the total proteins of the original serum. With such a high removal of the serum proteins a certain proportion of the antitoxic units must be carried down with the First Fraction precipitate instead of with the Second Fraction precipitate. The antitoxin thus brought down with the First Fraction precipitate can only be extracted therefrom with great difficulty; hence the apparent and inexplicable loss of antitoxin under the conditions of the concentration.

It having been previously noticed that the heat-denaturation induced in the prolonged heating of sera to 57° is considerably increased by the addition of phenol, trikresol and cresylic acid to the serum, an experiment was made to ascertain whether the same phenomenon was experienced in the shortened heating process of Banzhaf's method. As this was found to be the case the investigation was carried further to ascertain whether the increased precipitation was due to changes in the hydrogen ion concentration caused by the addition of these substances.

In Table VI have been embodied data to illustrate the influence of phenol and cresylic acid on the precipitation of the serum proteins during the heating of the serum-ammonium-sulphate mixtures by the Banzhaf (1913) method. Similar results have been obtained by other workers in this laboratory from the use of trikresol, ether and chloroform.

From a study of the table it will be seen that while the addition of the above-mentioned substances had no appreciable effect on the hydrogen ion concentration of the sera, there was a marked increase in the precipitation

of protein during the heating and at the same time a marked improvement in the filtration of the hot serum mixtures. The end products were characterised by their clearness and their ease of filtration; they contained no euglobulin.

TABLE VI.

Showing the increased precipitation of serum proteins induced by the addition of phenol and its homologues to the serum-ammonium-sulphate mixtures previous to their being subjected to the shortened heating process of the Banzhaf (1913) Method.

Percentage of phenol or its homologues added to the plasma diluted with one-third its volume of water	p_H^+ of the carbolised plasma measured by the electrical method	p_H^+ of the carbolised plasma made 30% of saturation with ammonium sulphate	Percentage conversion of soluble into insoluble protein during the heating process	Remarks on the filtration of the hot serum-ammonium-sulphate mixtures	Loss of antitoxin during the heating process
I. *Plasma containing* 8·74 % *of protein.*					
0·0 phenol	7·748	7·347*	6·3	slow, filtrate opalescent	nil
0·1 „	7·730	7·035†	16·5	more rapid, filtrate slightly opalescent	„
0·2 „	7·730	7·034	23·0	rapid, filtrate clear	„
0·3 „	7·730	7·085	24·0	„ „ „	slight
0·4 „	7·730	7·085	26·0	„ „ „	nil
0·5 „	7·730	7·061	33·0	„ „ „	slight
II. *Plasma containing* 7·92 % *of protein.*					
0·0 phenol	7·460	—	2·0‡	slow, filtrate opalescent	
0·1 „	7·347	—	17·0	rapid, filtrate slightly opalescent	
0·2 „	7·347	—	24·4	rapid, filtrate clear	
0·4 „	7·347	—	27·9	„ „ „	
0·5 „	7·347	—	30·8	„ „ „	
III. *Plasma containing* 10·03 % *of protein.*					
0·0 cresylic acid	7·000	—	1·0	slow, filtrate opalescent	nil
0·35 „ „	7·000	—	27·0	rapid, filtrate clear	„
IV. *Plasma containing* 7·88 % *of protein.*					
0·0 cresylic acid	7·648	—	5·0	slow, filtrate opalescent	
0·30 „ „	7·648	—	26·0	rapid, filtrate clear	

* p_H^+ of the saturated solution of ammonium sulphate added to the plasma = 6·480.
† „ „ „ „ „ „ = 6·010.
‡ „ „ „ „ „ „ = 5·900.

As the addition of phenol, etc., to the serum mixtures previous to their being heated does not cause any alteration in the hydrogen ion concentration it is obvious that the improvement in filtration is due to the specific action of these substances on the proteins themselves, although the mode of action is different from that of sodium chloride. The latter merely serves to stimulate the aggregation of the particles of already precipitated protein, the former induce a considerable increase in the precipitation of the serum

proteins by 30 % of saturation with ammonium sulphate, thereby assuring the complete precipitation of euglobulin at this stage. It is quite possible that the increased heat-denaturation of the serum proteins is brought about by an effect of these substances on the surface tensions of the colloidal particles of dissolved protein whereby their precipitability is considerably increased. Whatever may be the fundamental cause of this change the fact remains that the effect is so marked that, not only is the precipitating power of 30 % of saturation with ammonium sulphate considerably increased, but, in the cases investigated, the agglutination of the aggregates of the precipitated protein is so well defined that the reaction of the serum mixtures is of secondary importance. Moreover, the proteins involved do not seem to enter the intermediate ill-defined stage between the emulsoid and the suspensoid state which proves so great a hindrance to the filtration of the serum mixtures and of the final products.

From the data embodied in Table VI we see that, by the addition of phenol or its homologues, the heat-denaturation of the serum proteins can be regulated to the extent suitable for a particular serum.

For those who prefer to use the Banzhaf method this expedient is recommended until a simple routine method for the adjustment of the reaction of the serum mixtures previous to their being heated has been worked out. The judicious addition of 0·35 to 0·5 % of phenol or of 0·25 to 0·35 % of its homologues to the batches of plasma for concentration will result in the production of clear and readily filterable end products. If the addition of salt is also made a matter of routine, the concentration of the carbolised plasma or sera will not involve undue loss of antitoxin, although a considerable loss may result if the substances in question are added in amounts greater than those suggested.

(B) *The regulation of the factors causing irregular results in the concentration of sera by the more recent methods.* [Homer, 1916, 2.]

It has been found that there was slight trouble with the filtration of the end products from the concentration of sera in which the heat-denaturation had been less than 20 %, that is to say from the concentration of sera the reaction of which lay between the limits p_H^+ 5·4 and 7·0. In these cases the end products contained a small amount of heat-denaturated protein in colloidal suspension.

The occurrence of this difficulty could be prevented by a preliminary adjustment of the serum, previous to its being heated, to a hydrogen ion

concentration of about p_H^+ 7·8 to 8·0. It was inadvisable to increase the alkalinity further for the reasons given (*vide ante*, pp. 30, 31).

Trouble with the final filtration of end products from the concentration of sera of which the reaction fell within the limits p_H^+ 5·4 to 7·0 could also be obviated by the judicious addition of from 0·25 to 0·35 % of phenol or its homologues to the serum previous to the preliminary heating.

It is inadvisable to add the latter substances to more alkaline or to more acid sera than those falling between the aforesaid limits as there is the danger of thereby inducing so great an increased precipitability of the serum proteins that transference of antitoxin to the First Fraction precipitate will occur. Under these conditions there will be an apparent loss of antitoxin during the concentration as the extraction of the antitoxin thus carried down with the First Fraction precipitate is a matter of great difficulty.

It has also been found advisable to adjust the reaction of the serum-ammonium-sulphate mixtures previous to the second stage of the heating process (*ante*, p. 32). It may also be stated that at this stage of the heating the temperature of the serum mixtures is not raised beyond 59°.

From the consideration of the work detailed above it is evident that the reaction of the sera must be taken into account by those engaged in the concentration of antitoxic sera. On the reaction of the serum depends, not only the extent of the heat-denaturation of the serum proteins, but also the successful precipitation of the serum proteins by ammonium sulphate. Unless the reaction be regulated the necessary increased precipitation of the proteins cannot be ensured except by the addition of phenol, etc., to the serum. The latter course, in the hands of inexperienced workers may prove somewhat costly, as these substances favour the destruction of antitoxin.

Although the regulation of the reaction of the serum is proving of material value the possibility must not be lost sight of it being shown at some future date, that, in the concentration of antitoxic sera, there are other, hitherto, unsuspected factors which also influence the operations involved. In any case it is clear that the standardisation and regulation of a concentration process can only be attained by a thorough knowledge of the factors involved.

Summary.

During the course of the investigation it has been demonstrated that:

(1) In the Banzhaf (1913) method the uncertainties in filtration are due to two causes:

(*a*) That no cognisance has been taken of the reaction of the serum.

(*b*) That as the precipitating power of 30 % of ammonium sulphate is not appreciably increased during the heating process, a certain amount of euglobulin escapes precipitation with the First Fraction precipitate and appears in colloidal suspension in the final product.

(2) The uncertainties in the filtration of the hot serum-ammonium-sulphate mixtures in the above method can be obviated by an adjustment of the hydrogen ion concentration. The filtration can also be improved by the addition of sodium chloride to the mixtures, but, in this case, the improvement is due to a specific action of salt on the globulins.

(3) The complete elimination of euglobulin can be assured by:

(*a*) An adjustment of the hydrogen ion concentration of the serum mixtures to the point at which the desired increased precipitation is assured.

(*b*) Brine extraction of the Second Fraction precipitate containing the pseudo-globulin-antitoxin combination together with the small amount of euglobulin which has escaped precipitation with the First Fraction precipitate.

(*c*) Subjecting the serum to a preliminary prolonged heating at a temperature of 57—58° whereby the precipitability of the serum proteins is considerably enhanced.

(*d*) The addition of organic substances such as phenol and its homologues, ether or chloroform which increase the precipitation of the serum proteins, presumably, by virtue of their effect on the surface tension of the protein aggregates in colloidal solution.

(4) The extent of the heat-denaturation of the serum proteins during the heating of serum at 57° for several hours is also influenced by the hydrogen ion concentration of the serum and can be controlled by the adjustment of the latter.

(5) The denaturation induced by heat in alkaline sera apparently does not involve the same type of change as that induced in acid sera.

REFERENCES.

Banzhaf (1913). Collected Studies Research Dep. Health, New York, **4**, 230.
Homer (1916, 1). *Biochem. J.*, **10**, 10.
—— (1916, 2). *J. Hygiene*, **15**, 388.
—— (1917). *J. Hygiene*, **15**, 580.
MacConkey (1914). *J. Hygiene*, **13**, 467.

VI. FURTHER CONTRIBUTIONS TO THE TECHNIQUE OF PREPARING MEMBRANES FOR DIALYSIS.

By WILLIAM BROWN.

From the Department of Plant Physiology and Pathology, Imperial College of Science and Technology, London.

(*Received February 26th, 1917.*)

Introduction.

In a previous communication [Brown, 1915] a method was described by which a series of collodion membranes of differential permeability could be prepared. These membranes were for use in aqueous solution. It has been found that the method there described can be extended to the case of other substances (e.g. gelatin, agar, parchment, etc.) and to a large variety of liquids (ethyl alcohol, acetone, chloroform, benzene, etc.). Furthermore by a modification of the method, a series of graded gelatin membranes has been obtained which can be used in aqueous solution.

General Principle.

By way of illustration we may take the case of the collodion technique described in the paper already cited. When films of air-dried collodion (celloidin) are immersed in water, only a negligible imbibition of the latter takes place. When similar films are immersed in absolute (or nearly absolute) ethyl alcohol, a rapid imbibition takes place, the film swelling up, and, if sufficient alcohol be present, disintegrating to form a uniform solution. By immersing the films in an alcohol-water mixture, an intermediate state is reached in regard to imbibition by the collodion, of such a type that the higher the percentage of alcohol used, the greater the resultant imbibition. When the films so treated are placed in water and thoroughly washed, it is found that while a certain amount of shrinkage takes place in the process, the effect of the differential treatment is still strongly marked,

and thus, corresponding to a given strength of alcohol, a collodion membrane possessing a definite permeability and having water as its continuous phase may be prepared.

In the above illustration, the function of the water may be described as being to restrain the imbibition of alcohol by the collodion. For convenience in description we shall speak of "restraining" and "swelling" liquids, the former being exemplified in the above illustration by water and the latter by alcohol.

The general principle may now be enunciated:

Suppose a system consisting of:—Liquid *A*—Membrane substance—Liquid *B*.

If (1) the membrane substance only imbibes a negligible amount of *A*,

(2) the membrane substance strongly imbibes *B*,

(3) *A* and *B* are miscible in all proportions,

then by soaking films of the membrane substance in mixtures of *A* and *B* and subsequently washing in *A*, membranes of graded permeability can be obtained, and of such a type that the higher the percentage of *B* in the mixture the greater is the permeability[1] of the resultant membrane.

The membranes which have been produced by application of the above principle will be described under two headings:

A. Graded membranes for use in the restraining liquid, e.g. collodion in water, gelatin in alcohol. This class comprises the great bulk of the membranes prepared.

B. Graded membranes for use in the swelling liquid. Of this type, only one is at present known, viz., gelatin in water.

[1] In the paper already cited, the effect of the treatment with alcohol-water mixtures was shown by determining the change in the space relationships (e.g. increase in length, increase in thickness, diminution in percentage of dry to total weight), as well as by measurement of the rates of diffusion of particular substances through the membrane. In the case of the membranes dealt with in the present paper, it has not been found possible to carry out the experiments with the same amount of detail, partly on account of the large variety of membranes which have been produced, but mainly because of the impossibility of obtaining at the present time the glass-ware requisite for carrying out in the case of these membranes experiments on diffusion and dialysis. In the case of the collodion-in-water membranes it was found that high permeability was associated with a low dry weight/wet weight ratio, and *vice versa*, that is, that permeability increased with increased development of the external or continuous phase of the membrane. This correlation has been established in the case of gelatin-in-alcohol and gelatin-in-water membranes, and it is believed will be found to apply to membranes of all descriptions. In order therefore not to overstep the limits of experimentally ascertained fact, the statement in the text should be paraphrased into "the higher the percentage of *B* in the mixture, the lower is the dry weight/wet weight ratio of the resultant membrane, that is, the greater is the amount of its continuous (liquid) phase."

A. Graded Membranes for use in the Restraining Liquid.

Preparation of the Air-dried Films—Practical Notes.

Gelatin. 100 cc. of a 2 % solution of sheet gelatin in water (40—50° C.) is poured over a clean mercury surface in a shallow circular dish of about 20 cm. diameter and allowed to dry completely. The method of preparation by pouring over a flat glass surface was rejected on account of the difficulty of removing the dried film from the glass surface.

Agar. 100 cc. of a 2 % solution is poured over a flat horizontal glass plate (20 cm. diam.) and allowed to dry; when slightly moistened, the film may readily be peeled off. It is then soaked in alcohol (this involving a certain amount of shrinkage) and dried in a current of air. The method adopted in the case of gelatin was found to be impracticable in this case as the drying film invariably broke away from the sides of the vessel and dried with considerable shrinkage in area.

Starch. Only one membrane was prepared as it was found to be too brittle to possess any practical value. It was prepared in the same way as the gelatin membrane.

Collodion. The treatment is the same as in the case of agar films, the film being of course dried off without immersion in alcohol.

Preparation of Graded Membranes.

Films prepared as described above, together with vegetable parchment, were placed in various alcohol-water mixtures for 24 hours, and then transferred to absolute alcohol in which they were washed (with 3 changes) over a period of 2 days. The wet and dry weights of each membrane were then determined in the usual way and with the usual experimental precautions. The figures contained in Table I illustrate the type of result obtained.

TABLE I.

	(Dry weight/wet weight) × 100			
% Alcohol	Parchment	Starch	Gelatin	Agar
0	60·50	62·27	19·83	4·50
40	64·75	77·42	49·07	17·73
60	67·20	87·71	66·07	51·37
80	72·65	—	75·00	77·52
100	97·58	95·14	85·00	90·80

With regard to the above figures it must be remembered that in obtaining the wet weights of the membranes, it is impossible to remove completely the film of alcohol from the surface, and that therefore the figures obtained are all somewhat too low. The percentage error so introduced is obviously greatest in the 100 % alcohol membranes, and least in the case of those at the other end of the series. The error is greatest in the case of the agar and gelatin membranes as these were considerably thinner than the others.

The marked nature of the grading effected is sufficiently emphasised by the above figures not to require further comment. It is most pronounced in the case of the agar and gelatin membranes, and the latter have accordingly been selected as the most favourable substances for purposes of the present technique. Of these agar is preferred on account of the wider range it presents, and on account of the greater ease in its manipulation.

The figures obtained for parchment are anomalous in showing a very slow gradation over the greater part of the range of alcohol-water mixtures and a very sharp gradation towards the higher end of the series. This is considered to be due to the fact that parchment is not a homogeneous substance as it consists of two parchmentised layers with a paper layer between. The observed wet weight of a textile fabric like the latter is higher than the true wet weight as it includes the liquid which lies between the fibres and which is not properly speaking imbibed in the substance of the paper. The high figure obtained for the 100 % parchment membrane is due to the fact that such a membrane is impermeable to alcohol and therefore the internally situated paper cannot be reached by the latter.

The graded effect produced in these membranes can be readily demonstrated by observing the rate at which they stain on immersion in a dye solution. Thus when 40, 60, 80, 100 % membranes of parchment, gelatin and agar are placed in a strong solution of night blue in absolute alcohol for a short time (e.g. 15 minutes), taken out and rapidly wiped with a cloth to remove excess stain, it is seen that in all cases staining of the 100 % membranes is negligible, strong in the case of the 40 % membranes, with intermediate conditions in the case of the 60 and 80 % membranes. *Pari passu* with this graded staining effect runs the capacity of the particular membrane to transmit the dye, though this relationship has, on account of the difficulty of obtaining the requisite glass-ware, only been as yet fully ascertained in the case of the gelatin membranes. Thus in diffusion tests of alcoholic night blue against absolute alcohol with a series of graded gelatin membranes, no trace of the dye had passed through in 24 hours in the case of the 80 and 100 % mem-

branes; a slight passage occurred in the same time in the case of the 60 % membrane; whereas in the 40 % membrane strong coloration of the alcohol took place within a few (e.g. 5) minutes. A similar behaviour can confidently be predicted for the other membranes.

Uniformity and Permanency of the Grading Effect.

With films prepared in the same way the results obtained are markedly uniform. The figures in the following table give as usual the percentage of dry weight in total weight of membrane. The membranes were prepared simultaneously, and all washed in absolute alcohol for 3 days; the wet and dry weights of the first lot *A* were determined immediately, while the corresponding quantities for the second lot *B* were determined after a further 7 days. The ratio of the thickness when wet to original thickness when dry was also determined, and the results are also incorporated in Tables II and III.

TABLE II.

Agar.

	A (3 days in alcohol)		*B* (10 days in alcohol)	
% Alcohol	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$
0	4·50	ca. 1900	3·13	ca. 2200
	4·55	ca. 1900	5·18	ca. 1600
40	17·73	ca. 510	19·44	ca. 500
	17·65	ca. 500	18·05	ca. 500
60	51·37	183	53·57	172
	53·40	175	54·14	168
80	77·52	120	78·90	114
	78·22	127	78·94	117
100	90·80	100+	88·33	100
	90·23	100+	87·50	100

TABLE III.

Gelatin.

	A (3 days in alcohol)		*B* (10 days in alcohol)	
% Alcohol	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$
0	19·83	ca. 650	17·28	ca. 670
	19·30	ca. 630	18·53	ca. 700
40	49·07	220	52·21	235
	51·75	230	—	—
60	66·07	150	65·22	162
	64·28	150	66·67	160
80	75·00	120	74·47	123
	74·00	130	75·42	125
100	85·00	100	84·00	110
	—	—	—	—

It is thus seen that with similar treatment the membranes obtained show characteristic figures varying by not more than 2—3 per cent. With special precautions in the determination of these figures, it is certain that this range of error could be considerably curtailed.

Examination of the tables also shows that the membranes have not appreciably altered over a period of 1 week. More detailed tests by the staining method have failed to show any alteration over a period of 25 days. Similarly a 40 % gelatin membrane which allowed passage of the dye night blue in a few minutes was found to behave similarly after being kept for a period of 6 weeks. It is thus certain that these membranes preserve their properties with little alteration over a considerable period of time.

The degree of uniformity indicated in the preceding tables is not attainable unless the initial dry films have been prepared in a similar way. In the preparation of the solution from which the films are prepared, increased exposure to heat produces increased imbibition in the 24 hours' exposure to water and the alcohol-water mixtures, with corresponding differences in the dry weight percentage of the finished membranes. In the following table, a comparison is given of two films, one of which (*A*) was prepared by heating the original agar powder suspension to 100° for a few minutes; the other (*B*) by similar treatment at 120° for 20 minutes. It will be observed that the differential effect diminishes with increasing percentage of alcohol.

TABLE IV.

	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$		$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$	
% Alcohol	*A*	*B*	*A*	*B*
0	10·24	4·50	450	1900
40	41·28	17·73	195	510
60	58·93	51·37	152	183
80	75·33	77·52	115	120

The corresponding figures for the ratio of dry to wet weight (in water) after 24 hours' soaking in water are: *A*, 4·4 %; *B*, 1·5 %.

An exactly similar phenomenon occurs in the case of gelatin. This is in agreement with the statement of Wo. Ostwald [1905] that increased heating confers upon gelatin an increased power of imbibing water. It is obvious therefore that in carrying out the present technique the process of preparation of the original films must be standardised.

Generalisation. Graded membranes having been obtained by the above process, the absolute alcohol may be replaced by any liquid which is miscible

with alcohol and which is not imbibed by the dry gel substance. Thus in the case of agar and gelatin, absolute alcohol as continuous phase may be replaced by acetone, benzene, petrol ether, etc., and thus graded membranes prepared for use in these liquids. The process of preparation consists simply of washing out the alcohol by immersion of the membrane in several changes of the particular liquid.

Table V shows such a gradation effected in a series of agar membranes. The figures represent as usual the percentage of dry weight in total weight. All the membranes were prepared by soaking in alcohol-water mixtures, with subsequent transference to absolute alcohol. The experiment was performed in duplicate so that an idea might be obtained of the degree of uniformity realised.

TABLE V.

% of alcohol employed in grading	Eth. alc.	Acetone	Toluene	Benzene	Chloroform	Clove oil	Petrol
0	4·10	4·21	3·89	4·26	2·15	5·00	5·21
	4·23	4·42	3·84	7·93	2·28	5·24	4·49
40	17·88	20·27	18·63	25·00	11·60	25·15	26·55
	17·73	17·00	16·67	23·73	10·97	26·97	23·24
80	74·60	78·22	88·20	78·67	73·21	68·22	87·00
	74·08	76·92	88·42	79·00	74·42	68·60	88·90

It may be remarked that the figures in the horizontal rows of the above table are not comparable with each other. The low figures obtained in the case of the 0 % and 40 % membranes in chloroform are to be explained by the high specific gravity of chloroform. Even if the figures in the horizontal rows be corrected for the different specific gravities of the liquids concerned, it is not certain that they will even then be comparable, as it is possible that the volume of the continuous (liquid) phase of the membrane alters on transference from one liquid to another. An accurate investigation of this type has not yet been attempted.

A detailed determination of the permanence of the graded effect in these membranes has not been carried out. Membranes in clove oil and toluene have been kept for 6 weeks, at the end of which time the grading as far as could be judged from external appearances (thickness) was as pronounced as ever. It is tolerably certain that the permanency of grading demonstrated in the case of the membranes in absolute alcohol is characteristic of these membranes in general.

Membranes prepared by use of other Swelling and Restraining Liquids.

1. *Restraining Liquid.* Throughout the present section alcohol has been used as restraining liquid. Membranes of equal range can be obtained by a similar use of acetone.

In the earlier paper in which the technique of collodion membranes was described, water was employed to restrain the swelling action of alcohol. For this purpose it may be replaced by any other liquid which mixes with alcohol and which is not mbibed directly by the dry collodion. Thus graded collodion membranes have been prepared by usè of chloroform, benzene, toluene, and petrol ether as restraining liquids. The range of mixtures available varies in the different cases, the maximum concentration of alcohol which can be used in each case being shown by the following figures:

Alcohol in toluene... ...	30—40 %
Alcohol in benzene ...	40 %
Alcohol in petrol ether...	75—80 %
Alcohol in chloroform ...	90—95 %
Alcohol in water	97—98 %

It is probable that the best series of graded collodion membranes for use in such liquids as benzene and toluene can be prepared by previous manufacture in chloroform and transference to the particular liquid to be employed.

2. *Swelling Liquid.* The scope afforded by the method under consideration is well illustrated by the variety of graded series of membranes which can be prepared from the acetate derivative of cellulose[1]. This substance is soluble in acetone and acetic acid, and swells to a considerable extent in chloroform. It has been found possible to prepare graded membranes by use of any of the following mixtures:

Acetone—water,
Acetone—ethyl alcohol,
Acetic acid—water,
Chloroform—ethyl alcohol,
Chloroform—benzene,
Chloroform—toluene,
Chloroform—petrol ether,

the membranes after being placed in the mixture being finally set up in the

[1] This material was kindly supplied to me by Mr Cross of Messrs Cross and Bevan, New Court, Lincoln's Inn, W.C.

second liquid in each case. Whether, as is probable, these methods are of unequal practical value has not up to the present been determined.

While the method of grading put forward in the present paper is undoubtedly of very wide application, the writer is not prepared to state that where the conditions as laid down on p. 41 are fulfilled, a graded series of any particular membrane substance can always be obtained. Thus it is not certain that graded rubber membranes for work in alcohol can be prepared by use of benzene-alcohol mixtures. As far as preliminary observations went, it appeared that in all cases the differential treatment became obliterated when the membranes were washed in alcohol. The nature of the particular membrane substance therefore plays a part in determining the nature of the result obtained. It is noteworthy that all the substances with which the most marked success has been achieved—agar, gelatin, celloidin—are characteristically plastic substances, whereas rubber is eminently elastic. The difference in effects observed is believed to be correlated with this difference in respect of shear elasticity.

Again, as was stated in the earlier paper dealing with this subject, it was found impossible to produce any effective grading of celloidin by use of acetone-water mixtures. The range of mixtures here available was somewhat limited, 0—60 %, but in addition to that it was observed that the celloidin films towards the upper end of the series tended to disintegrate without the marked continuous swelling which is characteristic of similar films when placed in alcohol. The volume of the continuous phase of such a membrane up to the stage where it was still a manageable membrane was therefore too small to allow of its ever being converted into even a moderately permeable membrane.

At this point it may be opportune to summarise what is considered an ideal relationship in the system:—

Swelling liquid—membrane substance—restraining liquid,

from the point of view of the present technique.

Membrane substance—swelling liquid. The membrane substance should not go into complete solution, or if it does it should reach that state by a process of continuous imbibition. It should preserve a practicable degree of mechanical coherence up to a high degree of swelling. Only in such a case is it possible to prepare highly permeable membranes of practical utility.

Membrane substance—restraining liquid. The membrane substance should not take up anything beyond a trace of the restraining liquid. This condition is nearly realised in the case of celloidin in water, though not sufficiently so

to prevent the passage of water and the simplest salts. In the case of the cellulose-acetate membrane this condition is less closely realised, so that it is doubtful if this substance can be used for the preparation of so-called semi-permeable membranes.

B. Graded Membranes for use in the Swelling Liquid.

Though only one series of such membranes is as yet known (viz. gelatin in water), and though the range of permeability of these is comparatively limited, nevertheless they are considered to possess such practical and theoretical interest as to merit a detailed description. It is known at present for instance that these gelatin membranes can be applied with great effect in the purification of certain enzymes and that they show properties which indicate that they may prove of considerable use in the treatment of phenomena of selective permeability.

Theory of Method.

In all the processes hitherto discussed, the films of membrane substance, once a state of differential swelling had been established by immersion in the series of "swelling-restraining" liquid mixtures, were without further treatment transferred to the pure restraining liquid. It was obvious on theoretical grounds (and was abundantly verified by experiment) that were transference to take place to the swelling liquid, all the membranes would rapidly swell up to much the same degree, and thus the differential effect would be obliterated. If however while the membranes were still immersed in the series of mixtures, the capacity of imbibing the swelling liquid could be suppressed or at any rate reduced to a small amount, it was argued that after such treatment the membranes could be transferred to the swelling liquid without obliteration of the graded effect. The method adopted in the case of gelatin is as follows.

Films of air-dried gelatin are placed in a series of alcohol-water mixtures at 20° C. After 24 hours, a given quantity (1 cc. to 10 cc. of alcohol-water mixture) of 40 % formaldehyde is added to each. After 24 hours' treatment in this solution, the membranes are transferred to water and thoroughly washed; they are then ready for use.

It will be noticed that the addition of the formaldehyde somewhat alters the alcohol-water ratio in the mixture. It is obvious that the process could be carried out without this ratio being altered, but such a refinement of the method has not as yet been deemed to be necessary.

A typical series of results is set forth in the following table.

The gelatin consisted of the ordinary sheet gelatin (as supplied for manufacture of culture media). After treatment as indicated above, the membranes were washed for two days in distilled water.

TABLE VI.

Treatment (% alcohol)	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$
0	7·35
20	13·66
40	24·29
60	36·70
80	45·93
100	47·93

The grading may also be illustrated by measuring the increase in length and increase in thickness of the membranes. The following table gives the lengths and thicknesses of strips of commercial sheet gelatin after treatment according to the present technique, the corresponding quantity for the original aid-dried membranes being taken as 100 in each case.

TABLE VII.

Membrane	0 %	20 %	40 %	60 %	80 %
Length	112·5	105·4	103·3	102·2	102
Thickness	390	350	290	225	185

The grading is thus shown markedly by alteration in thickness of the membrane. The effect in lengthening the membrane is comparatively slight. In the case of membranes prepared from films which have dried under tension according to the method described on p. 42, an actual contraction due to the release of this tension has been observed when the membranes are soaked in the intermediate concentrations of alcohol.

By this technique membranes ranging from about 5 % to nearly 50 % of dry substance can be prepared. In no case has a membrane of greater content than 50 % in dry weight been obtained; in other words, it has been found impossible to formalise a membrane so strongly as to prevent it taking up on immersion in water at least an amount of water equal to its own weight. At the other end of the series a limit is placed by the increasing mechanical weakness of the membrane. Membranes of less content in gelatin can readily be prepared by carrying out the imbibition process at a somewhat higher temperature, but their extreme fragility renders them of little practical value.

Test of Uniformity. With similarly prepared dry films, and similar treatment, a very considerable degree of uniformity in the resultant membranes can be attained. This is illustrated in Table VIII which gives the results of an experiment carried out in triplicate.

TABLE VIII.

Alcohol %	0 %	20 %	40 %	60 %	80 %	100 %
$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	12·66	17·86	23·86	34·76	44·07	26·43
	13·05	18·08	23·69	34·60	44·09	26·87
	12·17	18·14	23·83	34·72	44·09	26·83

Throughout this series the maximum variation from the mean is less than 0·5 %. This degree of uniformity is however by no means attainable when gelatin films of different origin are employed. A comparison of Tables VI and VIII brings out the essential features of the variations experienced. In both cases crude sheet gelatin as supplied for the manufacture of culture media was employed, that in the latter series being of a different make from the former.

A considerable amount of experimental work has been carried out with a view to determining the range of variation and the means of experimental control. The results may be briefly summarised as follows.

Considerable variations may be shown at the lower end of the series (membranes prepared in water and 20 % alcohol) by different samples of gelatin. This difference must be ascribed to the previous history of the gelatin. Films prepared in the manner described on p. 42 show a higher degree of swelling in water and 20 % alcohol than is shown by the original commercial sheet gelatin itself. This sensitiveness of gelatin to previous heating has already been mentioned.

Within considerable limits, the figures obtained are independent of the initial thickness of the membranes.

In the middle region of the series (40, 60, 80 %) remarkable constancy in results is obtained, no matter what the type of gelatin employed may have been. Thus in several hundred measurements which have been made at one time or another with different samples of gelatin, a maximum variation of about 3 % only has been observed in the dry weight percentage of such membranes.

A remarkable variation is shown by different samples of gelatin in respect of the membranes soaked in 100 % alcohol. This feature is illustrated by

a comparison of Tables VI and VIII. In the former case, the membrane prepared in 100 % alcohol differs only slightly from that prepared in 80 % alcohol; in the latter table it has a much lower gelatin ratio. This is due to the fact that in the latter case only the outer layers of the membrane are formalised, the inner mass being highly swollen and unformalised. By lengthening the formalising period, this result can be avoided. In any case it may be noted that very little practical advantage is obtained by extending the series beyond the 80 % mixture.

Length of Imbibition Period. Though a slow imbibition in water and the lower concentrations of alcohol can be demonstrated over several days, nevertheless the effect so produced is so slight as to render an imbibition period of over 24 hours unnecessary.

Length of Formalising Period. It has been seen that in the case of membranes in 100 % alcohol, a period of 24 hours may be insufficient for formalising. In the case of membranes in 0—80 % alcohol, only a slight difference could be detected between membranes which had been formalised for 4 days and those which had had 1 day's formalisation. Thus 24 hours may be considered ample for the process. Table IX, giving a comparison of 6, 10, and 24 hours' formalising, shows that the period allotted (24 hours) cannot safely be reduced.

TABLE IX.

Formalising period	Membrane					
	0 %	20 %	40 %	60 %	80 %	100 %
6 hrs.	6·93	12·43	20·80	30·82	37·31	32·94
10 „	7·24	12·93	21·64	33·33	40·56	42·03
24 „	7·33	13·24	23·20	36·48	44·32	45·83

Permanence of Graded Effect. Unlike the membranes treated of in Section A, the membranes of the present section are known to undergo a definite and appreciable change when kept in water. This consists in a slow uptake of water, so that the figures representing the gelatin ratio undergo a steady decline. The amount of this effect will appear from Table X.

The membranes were prepared in the usual way, i.e. with 24 hours' imbibition in the alcohol-water mixtures, and 24 hours' formalising. The first wet weight readings were taken after 3 days; and then further readings taken at intervals of 7 days. From preliminary experiments it was known that the absolute dry weight of formalised gelatin films when kept in water does not appreciably alter. Therefore the relative gelatin content of the membranes at any time can be determined by measuring the wet weight at the particular time and the dry weight at the end of the experiment. The figures in the table are as usual the percentage ratio of dry weight to wet weight.

TABLE X.

Membrane	After 3 days	After 10 days	After 17 days	After 24 days	After 31 days	After 38 days	After 45 days	After 66 days
0 %	7·45	7·53	6·61	5·80	5·42	5·55	6·33	7·10
20 %	14·66	14·13	13·20	12·33	12·28	12·20	12·20	13·00
40 %	23·62	22·62	21·00	19·70	18·84	17·83	17·00	16·54
60 %	35·67	34·01	32·00	29·55	28·13	26·83	25·50	23·26
80 %	44·90	42·00	39·42	36·00	35·00	33·00	31·60	30·00
100 %	45·95	42·13	40·00	37·31	34·82	32·81	30·63	26·71

It will be seen that in the case of the 80 % alcohol membrane, the ratio of dry to wet weight decreases by about 2·5 % per week; that the weekly decrease in the case of the 40 % alcohol membrane is about 1 %. The membranes at the lower end of the series show a very gradual decrease. The slight increase shown towards the end of the experiment may have been due to a certain amount of bacterial contamination which appeared in the later stages of the experiment.

Attempts were made to reduce the rate of change indicated above, but so far without success. Thus membranes which were exposed for 24 hours to the action of formalin of full strength (40 % formaldehyde) in addition to the ordinary formalising treatment were found to behave in very much the same way as those which had only received the ordinary treatment. This property must therefore for the present be regarded as inevitable, and its presence must be remembered in all applications of these membranes.

The rate at which these membranes are degraded is considerably increased by the presence of acid and alkali. Tables XI and XII illustrate this effect.

TABLE XI.

Effect of Acid.

	Membranes in water		Membranes in $n/100$ H_2SO_4	
Membrane	After 1 day	After 15 days	After 1 day	After 15 days
0 %	7·91	8·22	7·60	5·56
20 %	13·00	12·50	14·87	10·62
40 %	24·67	22·30	23·00	15·39
60 %	34·67	30·35	34·74	21·77
80 %	42·53	35·70	42·57	28·40

TABLE XII.

Effect of Alkali.

	Membranes in water		Membranes in $n/100$ NaOH	
Membrane	After 1 day	After 15 days	After 1 day	After 15 days
0 %	7·28	7·73	7·00	5·00
40 %	22·74	21·00	24·00	15·63
60 %	37·44	33·04	37·46	24·67
80 %	45·80	40·00	46·00	32·18

With increasing strength of acid or alkali, it is highly probable that the rate of degradation is further increased. The instability of the membranes under such conditions renders them therefore unsuitable for work in acid or alkaline media. It may also be mentioned incidentally that membranes of gelatin cannot be used in the presence of acid pepsin, as they are rapidly attacked and totally destroyed.

General Remarks on the Application and Uses of these Membranes.

In applying these membranes to work on diffusion and dialysis it is to be kept in mind that in any series of membranes prepared from films of the same initial thickness, the more permeable members of the series are much thicker than those at the other end of the series. It will be observed that in this respect the technique of the present writer differs essentially from that described by Bechhold [1907]. The latter impregnated filter papers with solutions of various strengths of gelatin in water, or of celloidin in acetic acid, allowed to drain for a given time, and fixed, the former by immersion in 2—4 % formalin solution, the latter by immersion in water. It is obvious that by reason of viscosity the film which remains on the filter paper after drainage will be thicker in the case of the higher gel concentrations than in the case of the lower ones, and that therefore the apparent diminution of permeability of the membranes with higher gel content may be due in part simply to their greater thickness. If therefore the membranes described in the present paper be used in an investigation of the relationship between the "pore size" of a membrane and its capacity for allowing passage to substances in solution, it will be necessary to proceed in such a way as to ensure equal thickness in the resulting membranes. The initial dry films must thus be made correspondingly thinner when it is desired to use them for the preparation of the more permeable membranes. It is obvious that by a process of trial and error this experimental difficulty can readily be overcome.

The gelatin-in-water membranes described in the present paper compare favourably with the similar membranes prepared according to Bechhold's technique in respect of the much wider range (up to 50 % gelatin) of permeability and of the readiness by which they can be characterised. Bechhold's method was to standardise each particular lot in relation to its behaviour to a standard haemoglobin solution. The membranes of the present technique are standardised simply by determining the ratio of dry to wet weight of a

sample portion. The determination of this ratio is difficult if not actually impossible in the case of Bechhold's membranes on account of the presence of the supporting matrix of filter paper. It should also be noted that in Bechhold's technique, the concentration of gelatin in the final membrane may not be that of the gelatin solution originally employed. As the process of formalisation is a comparatively slow one, the gelatin membrane may swell considerably before that process is complete. It has for instance been found that membranes with as low a gelatin percentage as 5 % may be obtained by directly immersing air-dried films in 4 % formaldehyde at 20° C. Thus when in Bechhold's technique a filter paper impregnated with 10 % gelatin is placed in 4 % formaldehyde (even at 0° C.) it is not in the least certain that it formalises as such; on the contrary there is every probability that it swells during the process, producing a membrane of lower gelatin content and greater thickness. This consideration adds weight to the criticism as regards thickness emphasised above.

The method adopted by the present writer to characterise the collodion-in-water membranes was to state the concentration of alcohol in the mixture used in their preparation. In the case of the membranes dealt with in this paper (chiefly gelatin and agar), it has been shown that certain other factors (especially the thermal history of the gel substance) play a somewhat pronounced rôle. Unless therefore it be found possible and convenient to adhere to standard material, it would appear advisable to state, in addition to the alcohol concentration employed in the manufacture of the membrane, the ratio of dry weight to wet weight of the latter as determined on a sample portion.

Methods of attaching these membranes to glass-ware for purposes of diffusion and dialysis experiments will be described in a subsequent paper.

As regards the scientific or other interest of the large variety of membranes described in Section A of the present paper, it is obviously impossible to speak with certitude at the present stage, but it is plain that through them the scope of investigation by means of the processes of diffusion (in particular, dialysis) and adsorption is considerably extended. The present writer has begun an investigation by means of these membranes of the relation between the power of a membrane to adsorb a particular substance and its power to transmit it by diffusion, that is, of the relation existing between adsorption and permeability.

The practical aspects of this technique may also be mentioned. In the case of substances which are not readily separable from each other by dialysis

in water, it may be possible to find a solvent in which dialytic separation is practicable. An illustration of such is afforded in the case of Congo red and night blue dyes. Whereas no practicable separation can be obtained in aqueous solution, both substances being highly colloidal in water, the blue dye can be dialysed away with considerable ease in alcoholic solution, and thus a separation effected.

Lastly, these membranes can be used, not only in a large variety of liquids, but also in mixtures of the same. It is believed that in this feature there lies the possibility of an interesting investigation. Thus if a particular substance is crystalloidal in a particular solvent and colloidal in another, then it is probable that it shows intermediate characters in mixtures of the two solvents. It would thus be possible to vary the colloidal nature of the particular substance at will, and to determine its properties (diffusibility, degree of adsorption) in each case with a variety of membranes in equilibrium with the solvent mixture.

As regards the series of gelatin-in-water membranes treated of in Section B, it is already possible to speak with more definiteness. These membranes cover a portion only of the range covered by the collodion-in-water membranes, being, so far as is known at present, permeable to all electrolytes and to such simple colloids as safranin, neutral red, etc. As regards more colloidal substances, investigation has already shown that the order in which substances arrange themselves in reference to their diffusive capacity through gelatin is not the same as in the case of collodion membranes. One well authenticated case is afforded by the behaviour of starch and night blue solutions. It has been found possible to prepare a collodion membrane which completely prevents passage of night blue while definitely allowing passage to starch, whereas gelatin membranes have been prepared which allow slow passage of night blue but completely hold back a starch solution. It is hoped when this subject is further pursued that such a behaviour will be shown by a variety of substances (especially dye substances), so that a method may be evolved of attacking the problem of selective permeability in its purely physical relationships.

These gelatin-in-water membranes have been found to show properties which indicate that they may prove of service in the technique of enzyme purification. Membranes at the lower end of this series allow of the diffusion of the enzymes diastase and cytase, while those at the upper end hold them back. It is hoped to publish shortly an account of an investigation relating to the subject of enzyme purification.

Lastly, in relation to the phenomena of selective permeability, it would appear that certain dialytic processes can be carried out with greater effectiveness by means of these gelatin membranes than with membranes of collodion. Thus preliminary observations have shown that the dialytic separation of a starch-dextrin mixture can be carried out with greater rapidity with certain gelatin membranes than with any of the collodion series.

Summary.

The general principle by which membranes of graded permeability may be prepared is as follows:—

Suppose a system consisting of:—Liquid *A*—Membrane substance—Liquid *B*.

If (1) the membrane substance only imbibes a negligible amount of *A*,

(2) the membrane substance strongly imbibes *B*,

(3) *A* and *B* are miscible in all proportions,

membranes of graded permeability for use in the liquid *A* can be prepared by immersing films of the membrane substance in mixtures of *A* and *B* and transferring to liquid *A*. In this way are prepared graded membranes of collodion in water, chloroform, benzene, etc.; of agar and gelatin in alcohol, acetone, benzene, etc.; of cellulose acetate in water, alcohol, benzene, etc. Graded membranes for use in *B* can be prepared only when a method exists of suppressing the capacity of the membrane substance to imbibe *B*, this suppression to be brought about at the stage where the membranes are still immersed in the mixture of *A* and *B*. In this way graded membranes of formalised gelatin for use in water have been prepared.

REFERENCES.

Bechhold (1907). *Zeitsch. physikal. Chem.*, **60**, 257.
Brown (1915). *Biochem. J.*, **9**, 591.
Ostwald, Wo. (1905). *Arch. gesammte Physiol.*, **109**, 277.

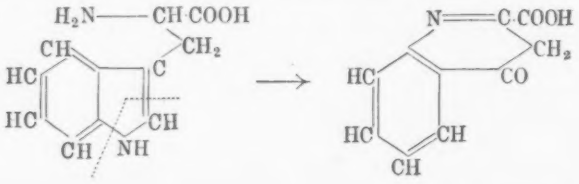
VII. THE SYNTHESIS OF RACEMIC Pr-2-METHYLTRYPTOPHAN.

By GEORGE BARGER and ARTHUR JAMES EWINS.

From the Department of Biochemistry and Pharmacology, Medical Research Committee, Lister Institute, London, S.W.

(*Received March 5th, 1917.*)

The mechanism of the changes involved in the complete oxidation of tryptophan in the animal body is quite unknown, partly no doubt on account of the difficulty of synthesising indole derivatives, which might be presumed to be intermediate stages in the degradation of this essential amino-acid. The only known product of its transformation is kynurenic acid which has so far been observed in the dog, the rabbit and the rat. (Note by F. G. Hopkins in a paper by Asayama [1916].) According to Homer [1915] and to Asayama kynurenic acid is not a product on the main line of the destructive breakdown of tryptophan, but in spite of this it is desirable that the mechanism of transformation should become known. "It is possible to picture the conversion of tryptophan to kynurenic acid in many different ways" [Dakin, 1912, p. 72]; the first question is whether the single nitrogen atom of kynurenic acid is represented by the indole- or by the amino-nitrogen of its precursor. Ellinger [1904], when he first identified the precursor, accepted the latter alternative, considering tryptophan to be α-amino-α-scatoleacetic acid; kynurenic acid was at that time regarded as being 4-hydroxyquinoline-3-carboxylic acid. The recognition of the true constitution of tryptophan rendered Ellinger's view less likely, until it was shown by Homer [1913] that kynurenic acid is 4-hydroxyquinoline-2-carboxylic acid. This correction of Camps' formula seems to us to re-establish a strong probability in favour of the elimination of the indole-nitrogen, as follows:



On the other hand it has been considered that the pyrrole ring is enlarged to a pyridine ring by the introduction of a carbon atom between the 2 and 3 positions, for which there is a good deal of analogy *in vitro*. Possible mechanisms have been discussed, e.g. by Dakin [1912], but the subsequent recognition of the fact that in kynurenic acid the carboxyl group adjoins the nitrogen atom, has made it necessary to assume, on this hypothesis, that the carboxyl group (oxidised side chain of tryptophan) wanders from the 3-to the 2-carbon atom, which is unlikely.

If by feeding a substituted tryptophan, a substituted kynurenic acid were obtainable, it would be possible to decide between the two hypotheses. A substituent in the benzene ring would be most suitable for this purpose, but is difficult to introduce, and we have therefore contented ourselves for the present with the preparation of a tryptophan methylated at the α-carbon atom of the pyrrole ring (*Pr*-2) which substance is relatively easily synthesised. We were led to this choice by the fact that 2-methylindole (methylketole) is the most readily obtainable indole derivative, and by the observation, that from this substance 2-methylindole-3-aldehyde is obtainable by Gattermann's hydrocyanic acid method in an unexpectedly good yield. From this aldehyde the synthesis followed closely that of tryptophan by Ellinger and Flamand [1907]. The yields were better throughout, probably owing to the protective action of the methyl group on the pyrrole ring, but this very circumstance seems to prevent the rupture of this ring in the animal organism, for, in the only feeding experiment undertaken so far, the urine contained an indole derivative, but neither kynurenic acid nor a similar substance. As we are unable to continue the work at present, we give the details of the synthesis below.

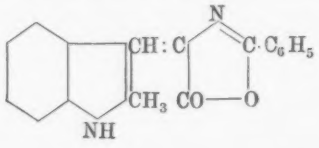
Preparation of 2-methylindole-3-aldehyde.

This substance has been previously obtained by Plancher and Ponti [1907] who acted on 2-methylindole with chloroform and sodium ethoxide, and by Angeli and Marchetti [1907], who obtained it from 2-methylindole and amyl formate, in the presence of sodium ethoxide. We find that Gattermann's aldehyde synthesis is applicable to the present case, and, as in the case of phenols, it is far preferable to the Tiemann-Reimer reaction. 21·5 g. of 2-methylindole was added to 150 cc. of dry ether, 19·2 cc. of anhydrous hydrocyanic acid and 21·5 g. of zinc chloride, freshly fused and powdered; hydrogen chloride was passed in, first for $2\frac{1}{2}$ hours at 0° and finally for $\frac{1}{2}$ hour at 45°. The almost colourless crystalline solid which had separated was filtered off,

washed with ether, boiled for a few minutes with water containing a little ammonia to neutralise traces of adherent acid, and filtered off after cooling. The crude product amounted to 23·9 g., and was somewhat pigmented. After recrystallisation from boiling xylene, in which the pigment is little soluble, we obtained a slightly coloured product melting at 202°; yield 20 g. or 75 % of the theoretical.

The aldehyde sublimes wholly at 180° in the vacuum of a Gaede pump without melting and when the resulting needles were crystallised from benzene flat acicular prisms were formed, M.P. 202—203°. Like Plancher and Ponti we also observed cube-like crystals.

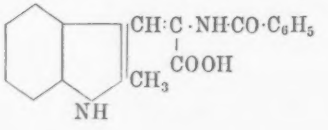
Azlactone of Pr-3-*Methylindolyl-α-benzoylamino-acrylic acid.*



17·3 g. of the aldehyde, 22·3 g. hippuric acid, 8·84 g. anhydrous sodium acetate and 39·3 cc. acetic anhydride were heated for 15 minutes on the water bath. The lactone began to separate when the solution was still hot. In a preliminary experiment the reaction product was extracted with boiling water, as indicated by Ellinger, but as we obtained hardly any unchanged aldehyde, we found it simpler to crystallise at once from glacial acetic acid, obtaining thus 30·5 g. of brownish red prismatic plates, melting at 204—207°. Ellinger and Flamand crystallised their lactone from chloroform, of which one molecule was retained as chloroform of crystallisation. Our lactone was not very soluble in chloroform and the air dry crystals retained an indefinite quantity, 10·6 %, rather less than half a molecule. The orange yellow glistening prisms so obtained sintered at 202°, and melted at 211°.

Analysis: 0·1318 dried at 100°; 0·3666 CO_2; 0·0580 H_2O. C = 75·8; H = 4·89.
Calculated for $C_{19}H_{14}O_2N_2$, C = 75·5, H = 4·63.

Pr-2-*Methylindolyl-α-benzoylamino-acrylic acid.*

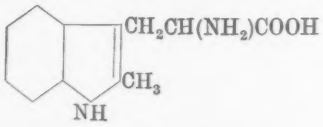


By repeated boiling with 100 times its weight of 1 % sodium hydroxide the lactone was hydrolysed to the above acid. After crystallisation of the precipitated acid from 70 % alcohol we obtained pale yellow prismatic needles, M.P. 221—222°, in a yield of 75 % of the theoretical.

Analysis: 0·1149 dried at 100°; 0·2994 CO_2; 0·0525 H_2O. C = 71·1; H = 5·1.

Calculated for $C_{19}H_{16}O_3N_2$, C = 71·2, H = 5·0.

Pr-2-*Methyltryptophan*



4 g. of the above acid were dissolved in 120 cc. of absolute alcohol and heated on the water bath with 12·5 g. of sodium in the course of half an hour. Then 20 cc. of absolute alcohol were added and the solution was boiled for 20 minutes, the flask being immersed in a salt bath. After adding 15 cc. of water, the removal of the benzoyl group was completed by a further boiling for 45 minutes. On pouring into 5 volumes of water the solution was washed with ether, acidified to Congo-red, left in the cold room over night, filtered, washed with ether and ethyl acetate, made up to 5 % with sulphuric acid and precipitated with Hopkins' reagent. After decomposing the mercury precipitate with hydrogen sulphide and treatment with lead carbonate in the usual way, about 60 % of the theoretical yield of an amorphous product was obtained, which yielded sphaerocrystals from 50 % alcohol, but could not be purified by crystallisation.

We therefore treated the concentrated aqueous solution of the substance with a slight excess of warm saturated picric acid solution in water. The gummy picrate which separated, afterwards became partially crystalline. It could be crystallised from picric acid solution (in water it dissociates) but better from methyl alcohol and petrol, which yielded orange red plates, melting at 173°.

Analysis: 0·1000 dried at 100°; 0·1775 CO_2; 0·0364 H_2O. C = 48·4; H = 4·0.

Calculated for $C_{12}H_{14}O_2N_2$, $C_6H_3O_7N_3$, C = 48·3, H = 3·8.

Regeneration from the picrate by means of sulphuric acid, washing with ether, and quantitative removal of the sulphuric acid by baryta was not successful. We therefore used the calculated quantity of nitron in dilute acetic acid solution. After filtration from the nitron picrate the solution was evaporated to dryness; the residue was dissolved in a little hot water, boiled with charcoal

and filtered. The amino-acid now crystallised satisfactorily. On recrystallisation from methyl alcohol and ether we obtained colourless prisms, melting variously at 263—273°, according to the rate of heating. The methyltryptophan crystallises with one molecule of methyl alcohol, which is retained *in vacuo* over sulphuric acid, but is given off at 110°.

Analysis:

0·1118 dried at 110°; 0·2861 CO_2; 0·0669 H_2O. C = 65·7; H = 6·25.

Calculated for $C_{12}H_{14}O_2N_2$, C = 66·05; H = 6·4.

0·1489 dried *in vacuo*, lost at 110° 0·0180 = 12·1 %.

0·0719 dried *in vacuo*, gave 0·1627 CO_2 and 0·0459 H_2O. C = 61·7; H = 7·1. $C_{12}H_{14}O_2N_2$, CH_3OH requires C = 62·4; H = 7·2; CH_3OH = 12·8 %.

Subsequently we found that the methyltryptophan could be crystallised at once from methyl alcohol and ether, if the crude substance was precipitated a second time by Hopkins' reagent. In this way we obtained a yield corresponding to slightly over 40 % of the theoretical.

Pr-2-Methyltryptophan is readily soluble in water, hardly soluble in absolute ethyl alcohol. It gives a strong reaction with triketohydrindene hydrate. With excess of bromine water a precipitate is produced, but no coloration. Perhaps we may conclude from this, that the bromine reaction of natural tryptophan consists in an attack on the (unsubstituted) α-carbon atom of the indole ring. Methyltryptophan gives with Hopkins and Cole's reagent a greenish blue coloration in dilutions up to 1 : 500. The reaction is therefore very much less sensitive than with the non-methylated amino-acid. In very concentrated solutions the ring test with sulphuric and glyoxylic acids gives at first a reddish purple coloration, which on mixing the two layers becomes deep blue.

The methyltryptophan we obtained has a sweet taste, probably due to its being racemic, for natural *l*-tryptophan is tasteless, *d*-tryptophan is sweet.

Behaviour of methyltryptophan in the dog.

0·5 g. of the substance, dissolved in 10 cc. of water, was given by a stomach tube to a puppy weighing 2 kilos[1]. The urine collected during 48 hours after the experiment, was purified by addition of half its volume of 10 % barium chloride containing 5 % ammonia, and kynurenic acid found to be absent,

[1] This experiment was kindly carried out for us by Dr H. H. Dale.

on estimation by Capaldi's method. The urine gave no reaction in the cold with glyoxylic and sulphuric acids, but on heating with 60 % sulphuric acid a dirty green colour was developed, not given by normal urine. We therefore removed the excess of barium, concentrated, acidified and extracted with ether, which solvent did not remove the substance giving the green coloration with hot sulphuric acid. We then evaporated to dryness and extracted the residue with ethyl acetate. The extract contained principally urea, but on destructive distillation of the residue, the vapours gave the pyrrole reaction with pine wood and the residue gave a reddish coloration with picric acid in aqueous solution. It appears possible therefore that methyltryptophan undergoes in the dog a change, which leaves the indole ring intact.

In a subsequent control experiment[1] the puppy was given 0·5 g. tryptophan and yielded 20—30 mg. of kynurenic acid, M.P. 280°.

Conclusion.

Further feeding experiments are required, but as long as they are negative it is impossible to draw definite conclusions as to the mechanism of the production of kynurenic acid. We think, however, that on the whole our result is in favour of the view that in natural tryptophan the pyrrole ring is eliminated, and that in methyltryptophan this elimination is prevented by the protective action of the methyl group. If the pyridine ring of kynurenic acid were formed by enlargement of the pyrrole ring, we might expect that methyltryptophan should yield a methylkynurenic acid, just as 2-methylindole yields 3-chloroquinaldine, when acted on by chloroform and alcoholic sodium hydroxide.

We desire to express our indebtedness to Mr W. W. Starling for valuable assistance in the preparation of the compounds described.

REFERENCES.

Angeli and Marchetti (1907). *Atti R. Accad. dei Lincei* [5], **16**, ii, 790.
Asayama (1916). *Biochem. J.*, **10**, 466.
Dakin (1912). *Oxidation and Reduction in the animal body* [Longmans, Green & Co., London].
Ellinger (1904). *Ber.*, **37**, 1801.
—— and Flamand (1907). *Ber.*, **40**, 3029.
Homer (1913). *J. Physiol.*, **46**. *Proc. Physiol. Soc.*, xviii and lxii.
—— (1915). *J. Biol. Chem.*, **22**, 391.
Plancher and Ponti (1907). *Atti R. Accad. dei Lincei* [5], **16**, i, 130.

[1] This experiment was kindly carried out for us by Dr H. H. Dale.

VIII. THE CONDITIONS OF ACTIVATION OF WASHED ZYMIN AND THE SPECIFIC FUNCTION OF CERTAIN CATIONS IN ALCOHOLIC FERMENTATION.

By ARTHUR HARDEN.

From the Biochemical Department, Lister Institute.

(*Received March 12th, 1917.*)

It was observed by Oppenheimer [1915] that the addition of a small amount of a pyruvate (1 per mille) or of pyruvic acid (1 %) to a mixture of Lebedev's maceration juice (prepared from dried yeast) and glucose caused an increase in the rate of fermentation of the glucose, and that a similar, but much smaller, acceleration was produced by the addition of acetaldehyde (1 : 200,000). Neuberg [1915, p. 75] somewhat later made a similar observation and extended it to the fermentation of fructose, mannose, cane sugar and maltose and also found [1915, p. 83] that other α-ketonic acids produced an analogous effect. He further attempted [Neuberg and Schwenk, 1915] to activate yeast-juice, which had been freed from coenzyme by dialysis, and zymin (acetone-yeast), rendered inactive by washing, by adding to them salts of individual α-ketonic acids and a phosphate. In this, however, he was unsuccessful, but by adding a mixture of salts of a large number of α-ketonic acids (pyruvic, α-ketobutyric, α-ketoisovaleric, α-ketocapronic, phenylglyoxylic, phenylpyruvic, *p*-hydroxyphenylpyruvic, hydroxypyruvic, oxalacetic and α-ketoglutaric) along with dipotassium hydrogen phosphate, he succeeded in obtaining a small amount of fermentation (of the order of 5 cc. of CO_2 from excess of sugar by the action of 20 cc. of inactivated maceration juice or 2 g. of inactivated zymin), and concluded that this mixture could in part replace the coenzyme.

On repeating these experiments it was found that zymin, which had been prepared from top-yeast and rendered inactive by thorough washing, was readily activated by potassium pyruvate in presence of a suitable concentration of a phosphate. The experiments were then extended to acetaldehyde, since this substance is the immediate product of the decomposition

of salts of pyruvic acid by the carboxylase of the zymin, which, as has been previously shown [Harden, 1913; Neuberg and Rosenthal, 1913] is not inactivated by washing. The first experiments were carried out in the presence of sodium phosphate and were unsuccessful, but it was subsequently found that, when the sodium phosphate was replaced by the potassium or ammonium salt, acetaldehyde activated the zymin in the same manner as the pyruvate.

It thus appears that potassium and ammonium ions play some special part in the process of fermentation in which they cannot be replaced by sodium ions. The presence of sodium ions is not in itself inhibitory, since activation occurs in their presence, provided only that potassium or ammonium ions are also present. This observation complements in an interesting manner the discovery by Adolf Mayer [1874] that potassium phosphate cannot be replaced by the sodium salt in a synthetic culture medium for yeast.

The fact that washed zymin can be activated by acetaldehyde in presence of a phosphate is of considerable interest from several points of view. In the first place it is consistent with, and may even be regarded as strong evidence in favour of, the theory now held by many investigators [for literature see Harden, 1914] that acetaldehyde is an intermediate product in alcoholic fermentation and is reduced in that process to alcohol by hydrogen liberated at a previous stage of the decomposition. According to this view the acetaldehyde would fulfil the function of an activator by serving as an acceptor for the hydrogen and would thus enable the reaction to start. It is possible however that other reducible substances would act in the same way and this would to some extent lessen the weight of the evidence in support of the acetaldehyde theory although it would be in agreement with the view that a hydrogen acceptor is a factor necessary for fermentation. This question will form the subject of further experiments as soon as circumstances permit.

These considerations lead directly to the question whether acetaldehyde can be regarded as constituting the coenzyme of yeast-juice. There is much in favour of this idea, but in view of the possibility that some other reducible substance may play the same part as acetaldehyde it is impossible to come to a definite conclusion without further investigation respecting the actual presence of acetaldehyde in yeast-juice and the activation of yeast-juice freed from coenzyme by dialysis, ultrafiltration and continued fermentation. As regards washed zymin, acetaldehyde certainly acts in all respects as a coenzyme, provided that phosphate and potassium or ammonium ions are present in suitable concentrations.

A second consideration is that the possibility of reactivating washed zymin by means of acetaldehyde and a phosphate affords a means of examining the effect of various cations and anions on the initiation and course of the fermentation and this opens a field of enquiry which may be expected to yield interesting results.

The stimulating effect of pyruvate and acetaldehyde on fermentation by yeast-juice observed by Oppenheimer and by Neuberg, also becomes of great interest in the light of these results and will form the subject of a further communication.

Experimental.

The zymin used in these experiments was prepared from a top-fermentation yeast employed in an English brewery by the usual method of treatment with acetone. It was washed five times with 6 parts of water in the manner previously described [Harden and Young, 1911] each washing lasting $\frac{3}{4}$ hour, and was then made up to a given volume with water. The same sample of zymin was used throughout the experiments quoted so that the results of experiments done with different batches are roughly comparable. All the fermentations were carried out in presence of toluene at 25°, the solutions being previously saturated with CO_2 at this temperature and incubated for 10—15 minutes before readings were made. The carbon dioxide evolved was collected and measured in the apparatus previously described [Harden, Thompson and Young, 1910] and the figures given are cc. of gas at atmospheric pressure and temperature.

Experiments with zymin.

I. The activation of washed zymin by potassium pyruvate and potassium phosphate and the effect of the replacement of these salts by the corresponding sodium salts.

14 g. of zymin were washed and made up to 80 cc.

In each experiment 10 cc. of this suspension (1·75 g. zymin) were used along with 1 g. of glucose and water to a total volume of 20·6 cc. To five quantities were added:

Exp. 1. 2 cc. 0·5 M K_2HPO_4.
„ 2. 2 cc. 0·5 M K_2HPO_4 + 3 cc. 1 % pyruvic acid (as K salt).
„ 3. 3·3 cc. 0·3 M Na_2HPO_4 + 3 cc. 1 % pyruvic acid (as Na salt).
„ 4. 1·6 cc. 0·3 M Na_2HPO_4 + 3 cc. 1 % pyruvic acid (as Na salt).
„ 5. 10 cc. boiled washings.

Time		Evolution of CO_2 in cc.				
h.	m.	1	2	3	4	5
1	5	0·5	12·6	1·9	2·1	11·1
2	5	0·5	20·4	2·1	2·8	17·6
20	20	2·9	55·8	2·5	6·6	69

In presence of the sodium salts (Nos. 3 and 4) little more CO_2 was evolved than in the control (No. 1) in the absence of coenzyme, whereas in the presence of the potassium salts (No. 2) the fermentation is almost as vigorous as in the presence of boiled washings (No. 5).

II. The activation of zymin by acetaldehyde and potassium phosphate or ammonium phosphate and the effect of the replacement of these salts by sodium phosphate.

A. *Exp.* 6. This was carried out along with the foregoing series. To the mixture of zymin glucose and toluene were added 1 cc. acetaldehyde solution (0·7 %) + 2 cc. 0·5 M K_2HPO_4.

Time		Evolution of CO_2 in cc.	
		Control	
h.	m.	No. 1 (above)	6
1	5	0·5	13·3
2	5	0·5	19·8
20	20	2·9	52·4

B. 10 cc. of washed zymin suspension and water to 20·6 cc. To six quantities were added:

Exp. 7. 10 cc. boiled washings + 1 g. glucose.
„ 8. 3 cc. 0·5 M K_2HPO_4 + 1 g. glucose.
„ 9. 3 cc. 0·5 M K_2HPO_4.
„ 10. 3 cc. 0·5 M K_2HPO_4 + 1 g. glucose + 1 cc. acetaldehyde.
„ 11. 2 cc. 0·5 M K_2HPO_4 + 1 g. glucose + 1 cc. acetaldehyde.
„ 12. 1·6 cc. 0·3 M Na_2HPO_4 + 1 g. glucose + 1 cc. acetaldehyde.

Time		Evolution of CO_2 in cc.					
h.	m.	7	8	9	10	11	12
	55	14·1	0·2	0·5	14·2	11·7	0·9
1	55	25·1	0·6	1·1	29·6	19·5	1·6
3	0	33·2	0·7	1·3	37·7	23·6	2·3
18	10	89	0·8	1·3	80·1	51·6	2·3

The amount of sodium phosphate added was only half the equivalent of the K salt because a previous experiment had shown that even in the presence of a small concentration of K ions, no fermentation occurred in presence of the full equivalent.

C. 10 g. zymin to 60 cc. 10 cc. of zymin suspension + 1 g. glucose were taken. Total vol. = 20·6 cc. To three lots were added:

Exp. 13. 10 cc. boiled washings.
„ 14. 2 cc. 0·5 M K_2HPO_4.
„ 15. 2 cc. 0·5 M $(NH_4)_2HPO_4$ + 1 cc. acetaldehyde.

Time		Evolution of CO_2 in cc.		
h.	m.	13	14	15
1	10	8·1	1·4	6·5
3	25	16·1	1·5	15·6
20	35	39·8	1·5	34·6

Here again acetaldehyde and potassium phosphate (Nos. 10, 11) or ammonium phosphate (No. 15) produce a good fermentation whereas acetaldehyde and sodium phosphate (No. 12) are without action.

III. Negative effect of potassium ions and acetaldehyde in absence of mineral phosphate.

Six quantities of 10 cc. of zymin suspension + 1 g. glucose + water to 20·6 cc. were taken and to these were added:

Exp. 16. 10 cc. boiled washings.
„ 17. 3 cc. 0·5 M K_2HPO_4 + 1 cc. acetaldehyde.
„ 18. 3 cc. M $KHCO_3$ + 1 cc. acetaldehyde.
„ 19. 3 cc. M $NaHCO_3$ + 1 cc. acetaldehyde.
„ 20. 1·6 cc. 0·3 M Na_2HPO_4 + 1 cc. acetaldehyde.
„ 21. 0·8 cc. 0·3 M Na_2HPO_4 + 1 cc. acetaldehyde.

Time		Evolution of CO_2 in cc.					
h.	m.	16	17	18	19	20	21
1	10	11·7	14·4	2·1	1·3	0·8	1·5
2	10	18	28·3	2·3	1·7	1·4	1·9
18	10	69	74·9	3·3	3·8	1·5	2

This shows that K ions and acetaldehyde in the absence of phosphate (No. 18) are incapable of producing fermentation and further that the same is true of Na ions (No. 19) and that the reduction of the concentration of sodium phosphate does not render it efficacious as an activator (No. 21).

Experiments with dried yeast.

Since dried yeast can be inactivated by washing [Euler and Bäckström, 1912] it can readily be used for experiments on activation. It is however not so satisfactory as zymin since, probably owing to the presence of a certain proportion of living cells, some samples yield irregular results. The yeast was dried in the air at 37° and then ground in a coffee mill. It was washed in a similar manner to the zymin.

IV. Effect of the pyruvates and phosphates of potassium and sodium.

20 g. yeast, washed four times and made to 100. 15 cc. yeast suspension + 1 g. glucose + 1 cc. toluene; water to 30·6 cc.; five quantities were taken.

Exp. 22. 5 cc. 1 % pyruvic (as K salt).
„ 23. 5 cc. 1 % „ „ + 1·5 cc. 0·5 M K_2HPO_4.
„ 24. 5 cc. 1 % „ „ + 2·5 cc. 0·3 M Na_2HPO_4.
„ 25. 5 cc. 1 % „ (as Na salt) + 1·5 cc. 0·5 M K_2HPO_4.
„ 26. 5 cc. 1 % „ „ + 2·5 cc. 0·3 M Na_2HPO_4.

Time		Evolution of CO_2 in cc.				
h.	m.	22	23	24	25	26
	20	2·6	9·7	15·6	13·1	1·4
1	10	5·6	16·2	22·8	20·2	2·7
2	5	6·7	24·3	—	—	3·9
3	5	7·1	34·1	—	—	5·2
19	20	9·9	71·1	—	—	8·6

Here the substitution of sodium for potassium salts (No. 26), in equivalent amounts has resulted in the complete absence of any evolution of CO_2 beyond

that in the control in the absence of phosphate (No. 22). That the presence of sodium ions is not seriously harmful is shown by a comparison of Nos. 24 and 25 with No. 23.

V. Effect of substituting sodium phosphate for potassium phosphate in presence of acetaldehyde.

The dried yeast was treated as in IV. 15 cc. yeast suspension + 1 g. glucose + 1 cc. toluene; water to 30·6 cc.

Exp. 27. 1·5 cc. 0·5 M K_2HPO_4.
„ 28. 1·5 cc. 0·5 M K_2HPO_4 + 1·4 cc. 1 % acetaldehyde.
„ 29. 2·5 cc. 0·3 M Na_2HPO_4 + 1·4 cc. 1 % acetaldehyde.

Time		Evolution of CO_2 in cc.		
h.	m.	27	28	29
1		1·5	25·5	2·9
3		1·5	41·8	3·2
5	30	1·7	58·3	3·8
21	45	4·4	86·3	6·1

The contrast here between the action of equivalent quantities of sodium and potassium phosphate is very marked.

VI. Effect of the addition of various chlorides in presence of sodium phosphate and acetaldehyde.

Dried yeast, treated as above. 15 cc. of yeast suspension + 1 g. glucose + 1 cc. toluene + 0·5 cc. 0·3 M Na_2HPO_4 + 5 cc. 0·1 % acetaldehyde; water to 30·6 cc. Four quantities.

Exp. 30. 1·5 cc. M KCl.
„ 31. 1·5 cc. M NH_4Cl.
„ 32. 1·5 cc. M NaCl.
„ 33. Control.

Time		Evolution of CO_2 in cc.			
h.	m.	30	31	32	33
1	15	7·7	7·8	0·3	1·9
2	15	10·6	11·1	2·5	2·8
18		11·6	13·4	3·3	3·3

This shows that whereas the addition of KCl (No. 30) or NH_4Cl (No. 31) to a mixture containing inactivated dried yeast, acetaldehyde and sodium phosphate causes a definite, though small, degree of fermentation, that of NaCl (No. 32) has no effect, the amount of CO_2 evolved being the same as in the control (No. 33).

SUMMARY.

1. In the presence of potassium phosphate, zymin and dried yeast, which have been inactivated by washing, can be activated by the addition of a pyruvate or acetaldehyde.

2. A specific difference in relation to alcoholic fermentation exists between the ions of sodium on the one hand and of potassium and ammonium on the other.

REFERENCES.

Euler and Bäckström (1912). *Zeitsch. physiol. Chem.*, **77**, 394.
Harden (1913). *Biochem. J.*, **7**, 214.
—— (1914). *Alcoholic Fermentation* (London, Longmans & Co.).
—— and Young (1911). *Proc. Roy. Soc.* B, **83**, 451.
—— Thompson and Young (1910). *Biochem. J.*, **5**, 230.
Mayer (1874). *Lehrbuch der Gährungs-Chemie* (Heidelberg).
Neuberg (1915). *Biochem. Zeitsch.*, **71**, 1.
—— and Rosenthal (1913). *Biochem. Zeitsch.*, **51**, 128.
—— and Schwenk (1915). *Biochem. Zeitsch.*, **71**, 135.
Oppenheimer (1915). *Zeitsch. physicl. Chem.*, **93**, 235.

IX. ON A NEW TYPE OF CHEMICAL CHANGE PRODUCED BY BACTERIA. THE CONVERSION OF HISTIDINE INTO UROCANIC ACID BY BACTERIA OF THE COLI-TYPHOSUS GROUP.

By HAROLD RAISTRICK.

From the Biochemical Laboratory, Cambridge.
Report to the Medical Research Committee.

(*Received March 29th, 1917.*)

The investigation of the products of the putrefactive decomposition of proteins, commenced by Nencki, Baumann, Brieger and E. and H. Salkowski, has more recently been continued by Ellinger, Ackermann and others, who have investigated the bacterial decomposition of the amino-acids. These decomposition products, varied in nature as they are, are rendered all the more interesting because of the very marked pharmacological and toxicological action exhibited by some of them, e.g. tyramine (*p*-hydroxyphenylethylamine and histamine (*β*-iminazolylethylamine).

By far the most common of all the chemical changes induced in amino-acids by bacterial action is simple decarboxylation, i.e. the production of an amine from an amino-acid by the loss of CO_2.

$$R . CH_2 . CH(NH_2) . COOH \rightarrow R . CH_2 . CH_2 . NH_2 + CO_2.$$

Ellinger [1900] proved that the bases putrescine and cadaverine described by Brieger owe their origin to the decarboxylation by putrefactive bacteria of ornithine and lysine respectively. By the use of the same bacteria Ackermann [1910, 1] obtained a 42 % yield of histamine (*β*-iminazolylethylamine) from histidine. His medium contained peptone, glucose, salts and histidine. Barger and Walpole [1909] isolated tyramine (*p*-hydroxyphenylethylamine) from putrid horse flesh and showed that it was derived from tyrosine by the loss of CO_2. More recently Sasaki [1914] obtained a 78 % yield of the same base by the action of *B. coli communis* on a medium containing tyrosine, glycerol, and inorganic salts. These are only a few of a number of similar

results. In fact almost all the amines which might be expected to result from the known amino-acids have been obtained by bacterial action.

Of somewhat less interest, since the products are usually non-toxic, is the change brought about by bacteria which is known as "deaminisation," i.e. the production of a saturated acid and ammonia from the amino-acid.

$$R . CH_2 . CH(NH_2) . COOH + H_2 \rightarrow R . CH_2 . CH_2 . COOH + NH_3.$$

Thus "deaminisation" in the usually accepted sense of the word *is accompanied by reduction*. Baumann [1879] found that when tyrosine is digested with putrefying pancreas it splits up into ammonia and *p*-hydroxyphenylpropionic acid. In their investigation of the constitution of tryptophan Hopkins and Cole [1903] subjected this amino-acid to the action of the Rauschbrand bacillus and of *B. coli communis*. In each case the bacteria, when grown *anaerobically* in a medium containing Rochelle salt and gelatin in addition to the amino-acid, produced an acid identical with Nencki's scatole-acetic acid, which was subsequently shown by Ellinger to be indolepropionic acid. In the research referred to above Ackermann [1910, 1] found in addition to histamine, a small amount of β-iminazolylpropionic acid. δ-Aminovalerianic acid, first isolated by E. and H. Salkowski [1883] from putrid fibrin, was shown by Ackermann [1910, 2] to be produced by the partial deaminisation of ornithine.

In all the cases quoted it will be noticed that, in addition to the amino-acid under observation the medium also contains large amounts of other organic substances, the presence and nature of which have probably an influence on the end-products. Thus Ackermann has shown that the bacterial decarboxylation of pure amino-acids is favoured by the presence of peptone in the medium. Mellanby and Twort [1912] also observe that the production of β-iminazolylethylamine from histidine by a bacillus, which they isolated from the alimentary canal, is inhibited if the medium is slightly acid or if it contains glucose.

The gradual breakdown of the side chain in amino-acids containing a cyclic nucleus, as seen in the bacterial decomposition of tyrosine and of tryptophan, also illustrates the marked effect of external conditions on the end-products. Baumann [1879, 1880, 1, 2] found that in the putrefactive decomposition of tyrosine *p*-hydroxyphenylpropionic acid is first formed, which in presence of air breaks down further into *p*-hydroxyphenylacetic acid, *p*-cresol and, to a less extent, into phenol. Hopkins and Cole [1903] found a similar breakdown with tryptophan. Under anaerobic conditions

both the Rauschbrand bacillus and *B. coli* gave an abundant production of indolepropionic acid and only slight traces of indole. Aerobically, however, the putrefactive bacteria, or *B. coli*, gave a good yield of indole and indoleacetic acid, traces of scatole, but no indolepropionic acid.

In commencing a study of the action of the bacteria of the Coli-Typhosus group on different amino-acids it seemed desirable therefore, in order to avoid any possible secondary reactions, not to introduce any organic compounds into the medium in addition to the amino-acid under observation. To this end the amino-acid—in this case, histidine—was dissolved in Ringer's solution (mammalian) and this medium used for inoculation. In such a simple medium the bacteria did not grow well but this difficulty was to some extent overcome by sowing thickly and by long continued incubation.

In the preliminary stages a medium of the following composition was made up:

Pure histidine hydrochloride equivalent to 0·2 % of the free base.

2 cc. per cent. of 0·04 % solution of phenolsulphonephthalein to act as indicator.

Ringer's solution.

Normal soda solution until P_H after boiling is 7·35.

This was tubed, sterilised, and inoculated with 24 hour old cultures (from agar slopes) of (i) *B. coli communis*, (ii) *B. typhosus*, (iii) *B. paratyphosus A*, (iv) *B. paratyphosus B*, (v) *B. enteritidis* (Gaertner), (vi) *B. dysenteriae* (Flexner). Even after ten days' incubation at 37·5° there was no change in the reaction of the tubes containing *B. coli*, *B. typhosus*, and *B. paratyphosus A*, though the other three organisms had produced a marked alkalinity, which became intense after 20 days' incubation. After this time *B. typhosus* showed a slight alkalinity but there was still no change in the reaction of the tubes containing *B. coli* and *B. paratyphosus A*.

In order to prove whether the alkalinity were due to amine production the experiment was repeated on a larger scale using *B. paratyphosus B* as the organism for inoculation. Experimental details are given later in the paper. A substance was isolated from the products of bacterial action, which gave a crystalline picrate. It was found impossible to isolate any β-iminazolylethylamine picrate from this, but it was shown to be the picrate of urocanic acid (β-iminazolylacrylic acid). This would arise from histidine according to the following equation:

$$C_3H_3N_2 . CH_2 . CH(NH_2) . COOH - NH_3 = C_3H_3N_2 . CH = CH . COOH,$$

i.e. *by deaminisation without subsequent reduction* to β-iminazolylpropionic acid. The production of an unsaturated acid from an amino-acid is a type of bacteriological change which so far as is known has not been previously observed.

The history of the occurrence of urocanic acid is very interesting. It was first isolated by Jaffé [1874] in 1874 from the urine of a dog—hence its name: greatly to its owner's chagrin the dog disappeared after some days and was never recaptured. Although Jaffé and others investigated the urine of a number of dogs and men it was not re-discovered until 1898 when Siegfried isolated it once more from a dog's urine [1898]. These are the only instances on record of its isolation from urine. Since it was continually present in both cases in fairly large amounts (2—3 g. per day) it appears that these two dogs presented a rare anomaly of metabolism. In 1912 Hunter [1912] was able to identify a substance which he obtained by prolonged tryptic digestion of caseinogen as urocanic acid, and by direct comparison with a synthetic specimen of β-iminazolylacrylic acid obtained by Barger and Ewins [1911] proved that the two were identical. Inasmuch as Hunter only obtained the acid from one tryptic digest—subsequent repetition of the experiment failing to give him any urocanic acid—he concluded that it had probably arisen from bacterial contamination although he was unable to observe its formation by any of the bacteria which he tried. The results described in this paper seem to bear out this presumption. It is also possible that the two dogs, whose urine contained urocanic acid, may have been suffering from some bacterial infection of this type, though Jaffé states that his dog was apparently perfectly healthy.

It now became of interest to prove whether this type of change is characteristic of *B. paratyphosus B* to the exclusion of the other members of the group and whether it is peculiar to the particular strain which was employed. Two other different strains of *B. paratyphosus B* have been tried and each of them brought about the same change. It was also shown that the property is possessed to a greater or less extent by the other members of the group, i.e. *B. coli*, *B. typhosus*, *B. paratyphosus A*, *B. enteritidis* (Gaertner) and *B. dysenteriae* (Flexner). The reaction is thus not restricted to those members which produce an alkaline reaction. In fact the largest yield of urocanic acid was obtained with *B. paratyphosus A*.

Experimental.

The composition of the medium for inoculation, which was the same throughout, was as follows:

2·7 g. pure histidine monochloride (= 2 g. of free base).
12·9 cc. normal soda, to neutralise the HCl of the histidine monochloride.
1000 cc. Ringer's solution (mammalian).

900 cc. of this medium were introduced into each of six litre flasks, and the whole sterilised by steaming for 45 minutes on three consecutive days. Agar slopes were sown over the whole surface with 24 hour pure cultures in tryptic broth, the bacteria used being (i) *B. coli communis*, (ii) *B. typhosus*, (iii) *B. paratyphosus A*, (iv) *B. paratyphosus B*, (v) *B. enteritidis* (Gaertner), (vi) *B. dysenteriae* (Flexner). After 24 hours' incubation the growth was washed off the agar slopes with sterile Ringer solution, emulsified by agitating with the platinum needle, and introduced into the flasks taking all precautions to prevent contamination. The growth from five agar slopes was added to each flask. The flasks were placed in an incubator at 37·5°, those containing the first three organisms being allowed to remain for 40 days, while the remainder were incubated for 28 days.

The investigation of the products of bacterial action was the same in each case. After sterilisation, the solution was filtered through kieselguhr, neutralised with HCl, and evaporated to dryness on the water bath. The dried residue was ground up, thoroughly extracted with boiling methyl alcohol, the alcoholic extract evaporated to dryness, and the residue taken up with a little water. This solution was then precipitated completely with saturated aqueous picric acid, the precipitated picrate filtered off and recrystallised once from a minimal amount of boiling water. A small amount was reserved for M.P. determination, and the greater part ground up with a little water (5—10 cc.), acidified strongly with conc. HNO_3 (about 2 cc.) and the picric acid completely extracted by shaking with ether. In this way the very slightly soluble nitrate of urocanic acid was obtained which was washed with alcohol and ether, dried, weighed, and converted into the free acid by the addition of one equivalent of pure sodium bicarbonate to a concentrated aqueous solution of the nitrate. The free urocanic acid readily separated out, was filtered off and recrystallised from hot water with the addition of a very small amount of animal charcoal.

In the case of all the six bacteria used, I succeeded in isolating the picrate and nitrate of urocanic acid and also free urocanic acid itself.

Urocanic acid picrate is only slightly soluble in cold water and crystallises well from hot water. When not quite pure it forms rosettes of needles, which on further recrystallisation change to well-defined golden yellow prisms. The M.P. varies considerably with the rate of heating and so for identification the M.P.S of the six different specimens were determined in pairs, M.P. tubes containing two different specimens being attached to the same thermometer. In each case the different specimens melted at 212—215° according to the rate of heating. Barger and Ewins give for the picrate of β-iminazolylacrylic acid, M.P. 213—214°.

In a later experiment from 13·9 g. of pure histidine monochloride submitted to the action of a different strain of *B. paratyphosus B* for 47 days there were isolated 3·4 g. of urocanic acid picrate (M.P. 212—213°).

Analysis: 0·1100 g. gave 18·5 cc. N at 19° and 752 mm. = 19·31 % N.

Calculated for $C_6H_6O_2N_2 . C_6H_3O_7N_3$, N = 19·07 %.

Urocanic acid nitrate. In each case this salt was obtained, which Jaffé and later Hunter consider characteristic of urocanic acid. It forms a white crystalline precipitate which under the microscope appears as "bent sickle-shaped plates whose ends appear to be frayed or eaten away. Frequently many such plates are combined to form cross- or rosette-shaped aggregates" (Jaffé).

The yield of the nitrate varied largely with the particular organism used, which may have been due to a number of factors—unequal inoculation, growth or duration of incubation. From 2·7 g. histidine monochloride (= 2 g. free histidine) in each case the following amounts of urocanic acid nitrate were obtained: *B. coli* 0·72 g.; *B. typhosus* 0·10 g.; *B. paratyphosus A* 1·30 g. (= 58 %); *B. paratyphosus B* 0·37 g.; *B. enteritidis* (Gaertner) 0·13 g.; *B. dysenteriae* (Flexner) 0·64 g.

The M.P., which also varies with the rate of heating, was determined in the same manner as that of the picrate. In each case the two samples melted simultaneously at 195—197° with explosive decomposition.

Barger and Ewins give for a synthetic specimen M.P. 198°.

Urocanic acid. The free acid crystallises readily from hot water. On allowing a hot solution to cool slowly long iridescent needles were obtained, while on cooling more quickly the acid crystallised as a mass of very fine needles. The M.P. of urocanic acid as given by different observers varies within wide limits. Jaffé gave 212—213°, Siegfried 229°, Barger and Ewins 235—236°, Hunter 231—232° (corr.). As Hunter observes, the M.P. varies

greatly with the rate of heating, which may explain the discrepancies. The six different specimens obtained were compared, in pairs, and were found to melt simultaneously at 226—230° according to the rate of heating. All the specimens gave the red coloration with sodium *p*-diazobenzenesulphonate and all reduced alkaline permanganate in the cold—proving the presence of an unsaturated linking.

Analysis:

0·1672 g.; 0·2543 g. CO_2; 0·0862 g. H_2O.

0·4763 g. dried at 110° lost 0·0988 g. H_2O.

0·1139 g. of substance dried at 110° gave 20·3 cc. moist N at 18·5° and 758·4 mm.

	Found	Calculated for $C_6H_6O_2N_2 . 2H_2O$
C	41·49	41·38
H	5·73	5·75
N	20·57	20·30
H_2O of crystallisation ...	20·74	20·69

SUMMARY.

Histidine (β-iminazolyl-α-aminopropionic acid) is converted into urocanic acid (β-iminazolylacrylic acid), in a medium consisting of Ringer's solution + histidine, by the bacteria of the Coli-Typhosus group, i.e. *B. coli communis*, *B. typhosus*, *B. paratyphosus A*, *B. paratyphosus B*, *B. enteritidis* (Gaertner), *B. dysenteriae* (Flexner). This is the first recorded instance of the bacteriological conversion of an amino-acid into an unsaturated acid.

It is a great pleasure to express my indebtedness to Professor F. Gowland Hopkins and to offer to him my sincerest thanks for his advice and encouragement during the progress of this research.

REFERENCES.

Ackermann (1910, 1), *Zeitsch. physiol. Chem.* **64**, 504.
—— (1910, 2), *Zeitsch. physiol. Chem.* **69**, 273.
Barger and Walpole (1909), *J. Physiol.* **38**, 343.
—— and Ewins (1911), *J. Chem. Soc.* **99**, 2336.
Baumann (1879), *Ber.* **12**, 1450.
—— (1880, 1), *Ber.* **13**, 279.
—— (1880, 2), *Zeitsch. physiol. Chem.* **4**, 304.
Ellinger (1900), *Zeitsch. physiol. Chem.* **29**, 334.
Hopkins and Cole (1903), *J. Physiol.* **29**, 451.
Hunter (1912), *J. Biol. Chem.* **11**, 537.
Jaffé (1874), *Ber.* **7**, 1669.
Mellanby and Twort (1912), *J. Physiol.* **45**, 56.
Salkowski, E. and H. (1883), *Ber.* **16**, 1191.
Sasaki (1914), *Biochem. Zeitsch.* **59**, 429.
Siegfried (1898), *Zeitsch. physiol. Chem.* **24**, 399.